THE EFFECTS OF HYPOXIA ON NEURONAL CELL SIGNALLING

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A thesis submitted in partial fulfilment of the
requirements for the degree of
Doctor of Philosophy

QUEEN MARGARET UNIVERSITY

2009

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LIST OF ABBREVIATIONS AND DEFINITIONS OF TERMS

Δ^9-THC	Δ^9-tetrahydrocannabinol
2-AG	2-arachidonoylglycerol
7TMRs	Seven transmembrane receptors.
AA	Arachidonic acid
AC	Adenylyl cyclise
ACEA	Arachidonyl-2-chloroethylamide
ACh	Acetylcholine
$A_{control}$	Absorbance of control solution
AD	Alzheimer's disease
AEA	Arachidonyl ethanolamine
AIDS	Acquired immunodefiency syndrome
AM251	1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Anandamide	*N*-arachidonoylethanolamide
ANTI	Antibody
ANOVA	Analysis of variance
ARs	Adenosine receptors
A_{sample}	Absorbance of sample solution
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
B_0	Maximum binding
B50 cells	Foetal cortical neuronal cells
BAD, Bax,	Pro-apoptotic genes and proteins of Bcl-2 family
Bak and Bok	Pro-apoptotic genes and proteins of Bcl-2 family
BCL-2	B-cell CLL/lymphoma 2
Bcl-2	A prototype of genes and proteins they produce.
bFGF	Basic fibroblast growth factor
BMA	British medical association
BrdU	Bromodeoxyuridine
Ca^{2+}	Calcium ions
$(Ca^{2+})i$	Intracellular calcium
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
CBD	Cannabidiol
CBN	Cannabinol
CCC	Controlled cortical contusion
cDNA	Complementary DNA
CHO cell	Chinese hamster ovary cell
cGMP	Cyclic guanine monophosphate
CNS	Central nervous system
CO	Carbon monoxide
CO_2	Carbon dioxide

CPP32/Caspase-3	Cysteine protease CPP32/Caspase-3
CP55,940	Cannabinoid agonist
CREB	cAMP response element-binding protein
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Pen-Thr-NH2] CTOP
$D_{1,2}$	Dopamine receptor subtype1 or 2
DAG	Diacylglycerol
DAMGO	D-Ala^2-MePhe^4, Gly-ol^5-enkephalin
DbcAMP	Dibutyryl cyclic adenosine monophosphate
DOR	Delta opioid receptor
DENN/MADD	MAP-kinase activating death domain
DHBA	Dihydroxybenzoic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPDPE	D-Penicilamine^{2,5} enkephalin
DSLET	D-ser2, O-Leu5-enkephaly-Thr
dUTP	Deoxyuridine 5'-triphosphate
EAE	Experimental autoimmune encephalopathy
ECACC	European Collection of Cell Cultures
EDRF	Endothelium-derived relaxing factor
eIF2alpha	Eukaryotic initiation factor 2alpha
ELISA	Enzyme-linked Immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated protein kinase
ERK1/2	Extracellular signal regulated protein kinases 1&2
FAK	Focal adhesion kinase
FAM	Focal adhesion molecule
FBS	Foetal bovine serum
G	Gravity
G proteins	Guanine nucleotide binding proteins
Gαβγ	Heterotrimeric G alpha, beta, gamma proteins (G protein complex)
Gα	Heterotrimeric G alpha protein
Gβγ	Heterotrimeric G beta, gamma protein
GABA	γ-amino butyric acid
GAPs	GTPase-activating proteins.
GDP	Guanine diphosphate
Gi	Gαi GTPγS binding proteins
Gi-GTP	Gαi GTPγS binding proteins
GIRK	G-protein-activated inwardly rectifying kinase
Gq-GTP	G protein alpha q
GPCRs	G protein-coupled receptors
GPR55	G protein-coupled receptor 55
GPR119	G protein-coupled receptor 119
Grb2-SOS	Growth factor- binding protein 2-son of sevenless
GRK	G protein receptor kinase
GTP	Guanine triphosphate
GTPase	Guanine triphosphatase
H^+	Hydrogen ions

H_2O	Water
H_2O_2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HCl	Hydrogen chloride
HEK Cells	Human embryonic kidney cells
HIF 1α	Hypoxia-inducible factor 1alpha
HIF 2α	Hypoxia inducible factor 2 alpha
HPC	Hypoxia preconditioning
HRP	Horse radish peroxidise
H_2SO_4	Sulphuric acid
HSP 70	Heat shock protein 70
HU210	A synthetic cannabinoid
IAP	Inhibitors of apoptosis
ICI 174,864	N-allyl2-Tyr- (alpha-aminoisobutyric acid)2-Phe-Leu-OH
ICI 199,441	2-(3,4-Dichlorophenyl)-N-methyl-N-[(1S)-1-phenyl-2-(1-pyrrolidinyl)ethyl] acetamide hydrochloride
IFN-γ	Interferon-gamma
IGFBP-1	Insulin-like growth factor binding protein 1
IgG-HRP	Immunoglobulin G-Horse radish peroxidase complex
IL-1β	Interleukin-1β
IL-6	Interleukin-6
iNOS	Inducible NOS
IP_3	Inositol 1,4,5-trisphosphate
IPSCs	Inhibitory postsynaptic currents
JIP 1	JNK-interacting protein 1
JNK	c-Jun N-terminal kinase
JUN	N-terminal kinase
JWH-015	A synthetic cannabinoid agonist
JWH-133	A synthetic cannabinoid agonist
K^+	Potassium ions
kDa	Kilodaltons
KOR	Kappa opioid receptor
LC	Lateral contusion
LDH	Lactate dehydrogenase
LHRH	Lutinising hormone releasing hormone
LPS	Lipopolysaccharide
LSD	Least significant difference
LTP	Long-term potentiation
mAChRs	Muscarinic acetylcholine receptors
MAO	Monoamine oxidase
MAPKs	Mitogen-activated protein kinases
MEK	A proline-directed serine/threonine protein kinase
mGluRs	Metabotropic glutamate receptors
MHC II	Major histocompactibility complex class II
MMP	Matrix metalloproteinase
MOR	Mu opioid receptors.
MPT	Mitochondrial permeability transition
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
Mr	Molecular weight
mRNA	Messenger RNA

MRX	Micro plate reader
MS	Multiple sclerosis
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyoxy phenyl)2-(4- sulfophenyl)-2H-tetrazolium, inner salt]
MTT	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
N_2	Nitrogen gas
NA	Noradrenaline
NAC	N-acetyl cysteine
nAChRs	Nicotinic acetylcholine receptors
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NB	Neuroblastoma
NCP	Nucleus caudatus putamen
NEURO 2A	A Murine neuroblastoma cell line
NGF	Nerve growth factor
NK	Natural killer
NMDA	N-methyl-D-aspartic acid
NMDA	*N*-methyl-D-aspartate
NMDAR	NMDA receptor
nNOS	Neuronal form of nitric oxide synthase
NO	Nitric oxide
NOR	nor-Binaltorphimine dihydrochloride
nor-BNI	nor-binaltorphimine
NOS	Nitric oxide synthase
NSP	Non-specific binding
O_2	Oxygen
O_2^-	Superoxide anion
OH^-	Hydroxyl radical
ONOO-	Peroxynitrite
P42(MAPK2)	Extracellular signal-regulated kinase 2
P44(MAPK1)	Extracellular signal-regulated kinase 1
P450	Cytochrome monooxygenase enzyme
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PD	Parkinson's disease
PDE	Phosphodiesterase
PERK	Phospho endoplasmic reticulum kinase
PES	Phenazine ethosulphate
Phospho-ERK1/2	Phosphorylated extracellular signal-regulated kinase 1&2
PI	Phosphoinositol
PIC	Protease Inhibitor cocktail
PIP_2	Phosphatidylinositol bisphosphate
PI-PLC	Phosphatidylinositol-specific phospholipase C
PKA	Protein kinase A
PKB	Protein kinase B/Akt
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLCβ	Phospholipase C beta

PMSF	Phenylmethlsulfonyl fluoride
PN	Peroxynitrite
PNS	Peripheral nervous system
PPARS	Peroxisome proliferator-activated receptor
PTKs	Protein tyrosine kinases
PTX	Pertussis toxin
Raf	Serine/threonine kinases
Rap1	Repressor activator Protein1
Rap-GAP II	GTPase accelerating proteins 2
Ras, rap, ran	Small G proteins function as molecular switches
RGS	Regulators of G-protein signalling
Rho	Small G protein, a GTPase, a Ras superfamily.
RIPA	Radio-Immunoprecipitation Assay Buffer
RNA	Riboxynucleic acid
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
Rpm	Revolution per minutes
RT-PCR	Reverse transcription polymerase chain reaction
RyR	Ryanodine receptor
SA	Salicylate
SAG	Superoxide anion generation
SAPKs	Stress-activated protein kinases
SD	Sprague-Dawley
SD	Standard deviation
SK-N-SH	Human neuroblastoma cell line
SN	Substantia nigra
SNP	Sodium nitroprusside
SOD	Superoxide dimutase
TA	Total activity
TBARS	Thiobarbituric acid reactive substances
TBE	Tris/Borate/EDTA
TEA	Tetraethyl ammonium
THC	Tetrahydrocannabidinol
TMB	3,3',5'5-tetramethylbenzidine
TMEV	Theiler's murine encephalomyelitis virus
TNF-α	Tumour necrosis factor-α
tRNA	Transfer RNA
TRPV1	Transient receptor potential vanilloid 1
Trypsin-EDTA	Trypsin Ethylene diamine tetracetic acid
TUNEL	Terminal transferase dUTP nick end labeling
VEGF	Vascular endothelial growth factor
VTA	Ventral tegmental area
Win	Win 55,212-2 mesylate, a synthetic cannabinoid

LIST OF APPENDICES

DECLARATION

I hereby declare that I am solely responsible for the research reported in this thesis and the composition of the thesis.

Augustine Ibegbu

ACKNOWLEDGEMENTS

I would like to thank my Director of studies Dr Douglas McBean and members of my supervisory team Dr Lorna Fyfe and Dr Ian Mullaney for their continual guidance, encouragement and advice throughout the period of my studies.

My gratitude goes to Queen Margaret University, Edinburgh for the award of the Martlet Scholarship and the Ahmadu Bello University Zaria-Nigeria for awarding me a study fellowship which enabled me to undertake this research studies. I would also like to thank Promega Corporation for providing me with free samples of viability and proliferation assay kits and reagents.

My thanks goes to Dr Elizabeth Fashola-Stone, Technical Manager European collection of cell cultures (ECACC), for providing specialist and technical advise on the use of B50 cells. My appreciation and thanks goes to Dr Sanj Kumar, Vice President and General Manager, Europe, Discoverx Corporation Ltd and Anne Fowler, Application Specialist, Discoverx Corporation Ltd Birmingham for their kind advice on the method and use of cAMP assay. I wish to thank Dr Linda Ferrington of the Centre for Neuroscience, University of Edinburgh for her help and guidance in preparing RT-PCR.

Many thanks go Dr Emad Aldujaili for his help with the microplate reader. My appreciation goes to Ken Aitchison, senior technician, Shirley Coyle and all the technical staffs of the Department of Dietetics, Nutrition and Biological Sciences, for their help in dealing with many technical problems, with research equipments and ordering and storing of the reagents. I would like to thank all members of staff of the department and postgraduate students in Room 416 for all their support.

My thanks go to all members of my family especially my wife, Carol, my mum and my brothers Amarda and Bonny for their support, both morally and financially.

KEY WORDS: Hypoxia; Neuronal B50 cells; Cannabinoid receptor agonists; Opioid receptor agonists; neuronal signalling; Neuronal damage and protection

Abstract

Hypoxia adversely affects cells and tissues, and neuronal cells in particular have been shown to be more susceptible to the injurious effects of hypoxia i.e. they may begin to die when oxygen supply is reduced or completely eliminated. Cannabinoid (CB_1) receptor and opioid (μ, δ and κ) receptor agonists have been shown to elicit several central nervous system (CNS) effects, mediated via G protein-coupled receptors. The aim of the research presented in this thesis was to study the effect of hypoxia on neuronal cell signalling and the consequent neuroprotectant effects of cannabinoid and opioid receptor agonists against hypoxia in the rat cortical neuronal cell line (B50) in culture. The B50 cells cultured in hypoxic conditions were treated and concurrently cultured with cannabinoid and opioid receptor agonists to determine the effects of these drugs on hypoxia-induced changes using downstream signalling activities such as cellular morphogenesis, growth, proliferation, differentiation, lactate dehydrogenase (LDH) leakage, second messenger (cAMP) and extracellular signal-regulated kinases (ERK1/2) quantification, to assess the level of cellular damage and injury, repair and protection. Cortical B50 cells were cultured in either a normal incubator (21%O_2; 5% CO_2) as the normoxic control group, or a hypoxic incubator (5%O_2; 5% CO_2) as the experimental group. Three cannabinoid agonists [Win55,212-2 mesylate (Win), anandamide or arachidonoylethanolamide (AEA), and 2-arachidonylglycerol (2-AG)] and three opioid agonists [DAMGO (μ), DSLET (δ) and ICI-199,441 hydrochloride (κ)], were selected and administered to the cells as treatment group for 48 hours after 48 hours of initial culture for a total of 96 hours of culture and pre-treatment group treated at 0 hour for a total of 96 hours in hypoxic conditions at concentrations of 10nM, 50nM and 100nM for cannabinoid agonists, and 10μM, 50μM and 100μM for opioid agonists. Neuronal viability, proliferation, differentiation and second messenger activity were assessed using morphological same-field assessment, LDH leakage, cellular proliferation assay, second messenger (cAMP) assay, and phospho-ERK1 & 2 assay and dibutyryl cyclic adenosine monophosphate (DbcAMP) induced differentiation method. Levels of G-protein coupled receptor (cannabinoid, CB_1 and mu opioid, MOR) mRNAs were assessed using the RT-PCR method. The results showed that hypoxia induced a 4-fold increase in LDH leakage from B50 cells cultured in hypoxia when compared to the cells

cultured in normoxic conditions (440% versus 100%, respectively; $p<0.05$). Cannabinoid receptor agonist treatment was able to reduce the LDH release in hypoxic cells to between 2-to 4-folds: 100nM AEA (69%), 100nM 2-AG (103%) and 10nM Win (217%), when compared to untreated hypoxic B50 cells (440% versus cannabinoid treated; $p<0.05$). The results of opioid administration showed a 3-fold decrease in the level of LDH leakage in B50 cells cultured in hypoxia when compared to untreated hypoxic cells (587%). The results of hypoxic treated B50 cells with opioid agonists are 100µM ICI-199,441 (318%); 50µM DSLET (339%) and 50µM DAMGO (352%) ($p<0.05$; untreated hypoxia versus opioid treated). The result of cAMP quantification in B50 cells in culture showed a reduction in cAMP concentration in untreated hypoxic B50 cells when compared to normoxic cells (0.7 pmol/ml versus 3.0 pmol/ml; $p<0.05$). Cannabinoid treated hypoxic cells showed increases in cAMP concentration: 2-AG 10nM (3.5 pmol/ml), 50nM (3.1 pmol/ml) and 100nM (0.9 pmol/ml), ($p<0.05$; Cannabinoid treated versus hypoxia untreated). The cAMP concentration in B50 cells treated in hypoxia with opioid agonist, ICI 199,441 hydrochloride, was significantly increased when compared to untreated hypoxic B50 cells (0.7 pmol/ml). The treatment with ICI 199,441 hydrochloride are 10µM (10.0 pmol/ml), 50µM (3.15 pmol/ml) and 100µM (1.15 pmol/ml), ($p<0.05$; opioid treated versus hypoxia untreated). The result of phospho-ERK1&2 assay in B50 cells showed decrease in phospho-ERK1&2 in untreated hypoxic cells when compared to normoxic untreated cells (6.0 units/ml versus 87.0 units/ml; $p<0.05$). The result of cannabinoid treated hypoxic cells showed increases in phospho-ERK1&2 when compared with the hypoxic untreated B50 cells: Win 10nM (98 units/ml), Win 100nM (27 units/ml), AEA 10nM (62 units/ml), AEA 100nM (60.5 units/ml), 2-AG 10nM (45 units/ml) and 2-AG 100nM (68 units/ml) (cannabinoid treated versus untreated hypoxia; $p<0.05$). The phospho-ERK1&2 in hypoxic B50 cells treated with opioid showed increase with DAMGO 10µM (22 units/ml), DSLET 10µM (16 units/ml) and ICI 199,441 hydrochloride 10µM (23.5 units/ml) ($P<0.05$; opioid treated versus hypoxia untreated). The result showed a decrease in cellular proliferation in untreated hypoxic cells when compared to the normoxic cells ($7x10^6$ cells/ml versus $20x10^6$ cells/ml; $p<0.05$), while cannabinoid and opioid treatments was able to increase cell proliferation in hypoxic treated cells with: Win 10nM ($11x10^6$ cells/ml), AEA 100nM ($12x10^6$ cells/ml) and 2-AG 100nM ($13.8x10^6$ cells/ml), DAMGO 10µM ($16x10^6$ cells/ml), DSLET 10µM ($20x10^6$ cells/ml) and ICI

199,441 100μM ($21.5x10^6$ cells/ml) when compared to hypoxic untreated cells ($7x10^6$ cells/ml) (hypoxia untreated versus hypoxia treated; $p<0.05$). Some of these changes were shown to be concentration-dependent between the normal and hypoxic B50 neurons, and between treated and untreated hypoxic B50 cells in culture, while the CB_1 and MOR mRNA levels showed no appreciable change. The results show that B50 neuronal cells are susceptible to damage and injurious effects of hypoxia, as are most brain cells, while the results of the administration of cannabinoid and opioid agonists suggest that these agents have some potential therapeutic and protective benefits in the treatment and prevention of hypoxia-induced toxicity in neuronal B50 cells in culture. This could be of potential benefit in the treatment and protection against hypoxia-related neurodegenerative diseases and disorders such as stroke, dementias, ageing, Alzheimer's and Parkinson's diseases.

CHAPTER ONE

1. Introduction

1.1 General Introduction

Hypoxia refers to a condition in which there is a decrease in the supply of oxygen to any cell or tissue (Mishra et al., 2006; Feriche et al., 2007). Neural and brain tissues have been shown to be extremely sensitive to oxygen deprivation and begin to die when the oxygen supply is reduced or completely eliminated (Zhang, 2006; Guyton and Hall, 2005). A decrease in the exchange of respiratory gases in the lungs or in their transport in the blood can prevent the supply of oxygen from meeting the metabolic demands of the cells and tissues. This condition is called tissue hypoxia. Insufficient oxygen and nutrient supply have been shown to restrain tumour growth. Hypoxia-inducible factors (HIF) 1alpha and HIF 2alpha are transcription regulators of phenotypic adaptation to low oxygen levels (Jogi, et al., 2002; Airley et al., 2000; Semenza, 2000a; Semenza, 2001). When hypoxia lasts for extended periods of time it can lead to coma, seizures and even brain death. In brain death there is no measurable activity in the brain although cardiovascular activities and function may be preserved (NINDS, 2007; Coquelle et al.,1998; Guyton and Hall, 2005).

Hypoxia or oxygen deprivation can lead to oxidative stress which has been implicated in nerve cell death that occurs in a variety of neurodegenerative disorders like dementias, multiple sclerosis, Alzheimer's disease and Parkinson's disease (Maher, 2001; Benzi et al., 1994). Neuronal loss, neuritic and cytoskeletal lesions represent the major dementia-associated abnormalities in Alzheimer's disease (AD) (de la Monte et al., 2000). The loss of protein kinase C (PKC) activity has been coupled to the severity of the damage although the functional relationship between oxidative stress, protein kinase C and cell death is unknown (Maher, 2001).

Hypoxia leads to metabolic cellular processes in which oxidative species such as super oxide radical anions, hydrogen peroxide and lipid peroxides are generated intracellularly (Scandalios, 1997; Chen & Buck, 2000). These reactive species, if not eliminated, may damage DNA, proteins or membrane lipids and cause oxidative cell death. Endogenous antioxidative enzymes as well as endogenous small molecule

antioxidants are required for cells to survive (Scandalios, 1997; Chen & Buck, 2000; Semenza 2005), while exogenous small molecule antioxidants have been shown to effectively prevent oxidative cell death in cultured cells (Busciglio & Yankner, 1995; Nakao et al., 1996).

Hypoxic stress results in a rapid and sustained inhibition of protein synthesis that is partially mediated by eukaryotic initiation factor 2alpha (eIF2alpha) phosphorylation by the phospho-endoplasmic reticulum (ER) kinase (PERK) (Blais et al., 2004). Severe hypoxia has been shown to induce apoptotic cell death in developing brain neurons whereas mild hypoxia has been demonstrated to stimulate neurogenesis (Bossenmeyer-Pourie et al., 2002). Hypoxia threatens brain function throughout the entire life span starting from early foetal age until death and although the physiological consequences of brain hypoxia are well documented, the molecular mechanisms involved in these adaptive processes are still not well understood (Zhu et al., 2005; Rossler et al., 2001; Semenza, 2006; Semenza, 2007). It has been shown that hypoxia or reduced oxygen tension may have severe detrimental effects on most cells and especially on neuronal cells. Some studies have suggested that hypoxia can induce cellular adaptive responses that overcome apoptosis or cell death leading to reduced hypoxic cell injury, damage or cell death (Yun et al., 1997; Banasiak et al., 2000). These adaptive responses of cells to hypoxia may involve activation of some ion channels, as well as induction of specific gene expression which may help to suppress or limit the effects of hypoxia in these cells (Yun et al., 1997). For example, adenosine triphosphate (ATP)-sensitive potassium ion (K^+) channels are activated by hypoxia in some cortical cerebral neuronal cells, and this may play a role in cell survival during hypoxia (Yamada and Inagaki, 2002). This may explain why some cells may survive and adapt to the hypoxic environment than others. Also, hypoxia-induced basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) expression appear to be associated with prevention or delay of neuronal cell apoptosis in hypoxic conditions (Yun et al., 1997; Banasiak et al., 2000).

Gibson & Huang (2002), used the oxidative processes in the brain as a biomarker of Alzheimer's disease and showed that diminished metabolism and excessive oxidative stress occur in brains of patients with Alzheimer's disease. It has been observed that oxidative stress can cause neurodegeneration associated with enhanced susceptibility

to apoptosis due to the activation of proapoptotic genes (Gibson & Huang, 2002). Hypoxia-induced oxidative stress can also cause neurite retraction leading to neurodegeneration, while hypoxia-like injury can cause neuronal loss. Both oxidative stress and hypoxic injury could contribute to neurodegeneration similar to that found in Alzheimer's disease (de la Monte et al., 2000). It has been shown that ischemia results in severe focal and global damage of brain tissue accompanied by biochemical and molecular alterations, while hypoxia results in depletion of cellular and tissue energy and consequent death of the cells involved (Rodrigo et al., 2005).

Neuronal cell signalling is a specialized activity of the nerve cells through which they receive, integrate and transmit information referred to as signals within the cells and to the neighbouring cells. This is done by means of specific chemical stimuli or signals, cell-to-cell contact and through gap junctions. Chemical signal transmission is the major means by which nerve cells communicate within themselves and with one another in the nervous system (Siegel et al., 1999; Yu et al., 2001; Wenger, 2000). These signalling effects and mechanisms depend on the types of primary effectors to which the cells couple (Neubig & Thomsen, 1989). Thus according to Siegel et al. (1999), the first group of cells and effectors are those that have ligand-gated ion channels, the second group are the receptors with intrinsic guanylyl cyclase activity; the third group are those with intrinsic tyrosine kinase activity while the fourth group are the G-protein coupled receptors. The super family of G-protein-coupled receptors (GPCRs) is the main target for the actions exerted by hormones, drugs and neurotransmitters (Schmid et al., 2000). Each GPCR shows preferential coupling to certain members of the G-protein family (Gs, Gi or Gq) which in turn activates the defined second messenger pathways for example, via changes in the level of cAMP or protein kinase C activation or Ca^{2+} release from internal stores (Kukkonen et al., 2001; Nasman et al., 2002).

The G-protein-mediated signalling system has been widely used to study transmembrane signalling mechanisms in eukaryotic organisms (Wettschureck et al., 2004), which could result in many different cellular activities and effects such as cellular growth, proliferation and differentiation. Mammalian cells express many GPCRs as well as several types of heterotrimeric G-proteins and their effectors. The

G-protein-mediated signalling systems are made up of three main components (the receptors, the heterotrimeric G-proteins and the effectors) in addition to the various proteins that are able to modulate the G-protein-mediated signalling process like the regulators of G-protein signalling (RGS) proteins, which are able to adjust the sensitivity of the signal transduction system (De Vries et al., 2000; Neubig & Siderovski, 2002; Ross & Wilkie, 2002; Wettschureck et al., 2004). Most neurotransmitters of the central nervous system (CNS) act on GPCRs to mediate neuronal activity. These are present both presynaptically and postsynaptically and mediate slow response (Wettschureck et al., 2004). One group, the metabotropic glutamate receptors (mGluRs) couple to heterotrimeric G-proteins and regulate excitability and synaptic transmission at the glutamatergic synapses throughout the CNS (Saugstad et al., 1998).

Some studies have indicated that in a number of animal and cell models, activation of receptors that interact through Gi (i.e. group II mGlu and cannabinoid receptor types) appear to convey neuronal protection against hypoxic insult and resultant excitotoxic death (Bruno et al., 1994; Shen & Thayer, 1998; Rashidian et al., 2005). In this thesis, B50 neuronal cell lines derived from the neonatal rat central nervous system by Schubert et al. (1974), which have been widely used for the study of nervous system activities, were chosen as the model neuronal cell type. B50 cells have proved to be a useful tool for the study of neuronal cell differentiation, growth, toxicology and death (Otey et al., 2003).

1.2. Signalling Overview

Cells continuously receive information called signals from within themselves, their environment and from other neighbouring cells. The signals are converted into intracellular second messenger signals which ultimately result in a coordinated response by the cells. These responses could be in the form of cell growth and division, cell proliferation and differentiation, movement or even death. When cellular signalling is deregulated or altered, it results in the formation of cancer, deformed cells and hence diseases and cellular disturbances result. These cells not only have altered structure and morphology, they also have defective functions which further exacerbate the alteration in their signalling processes and mechanisms.

Cells make use of second messengers to bring about their signalling activities and functions (Haglund and Dikic, 2005). The second messengers required for the different cellular activities vary, depending on the final programmed cellular activity which determines the signalling input by the cell thus, resulting in an effective and programmed response of the cell to the stimuli (Heldin, 2003). Kinases have been shown to be important second messenger signals which add phosphate groups to cellular components and hence phosphorylate and energize them for their activities (Cohen, 2002).

Signalling activities are organised in pathways, and the beauty of cell signalling is the way different pathways are organised, combined and adapted to control a diverse array of cellular processes in widely different domains (Berridge, 2006). Many of the same signalling systems that control development come into play again to regulate a wide variety of specific activities in adult cells. These activities include cellular contraction, secretion, proliferation, information procession and sensory perception (Berridge, 2006), and these illustrate how cell signalling pathways are adapted and coordinated to regulate many cellular processes, mechanisms and functions (Hochstrasser, 2003).

Cell communication and signalling mechanisms occur either through electrical signals or chemical signals. Cell communication through electrical signals occurs in low-resistance gap junctions through which cells can communicate rapidly with each other by passing electrical current or through the diffusion of low molecular weight second messengers such as cyclic AMP, calcium ions, and inositol 1,4,5-trisphosphate (IP_3). In the case of chemical communication, one cell releases a transmitter or chemical agent, which diffuses to a target cell that has receptors which detect the transmitter or chemical agent and relay the information along various cell signalling pathways. This results in the activation of effectors in the cytoplasm or nucleus within the target cells to effect the desired response (Ben-Shilomo et al., 2003). These responses could be in the form of cell migration, pattern formation, specification of cellular fates and differentiation which enhances and establishes cellular structural and morphological identity and functional activity in the different cells and tissues (Haglund and Dikic, 2005; Berridge, 2006). Cell communication through electrical signals only occur in excitable tissues like neurons and muscles while communication through chemical

signals occur in all cells and tissues and make use of chemical stimuli such as neurotransmitters, growth factors and hormones (Berridge, 2006).

The basic principle of a cell signalling pathway involves the sending of stimuli like hormones, neurotransmitters or growth factors which act on cell surface receptors to relay information through intracellular signalling pathways, that can have a number of components to bring about the desired response. Cell signalling usually begins with the activation of transducers which use amplifiers to generate internal messengers that either act locally or can diffuse throughout the cell. These messengers then engage sensors that are coupled to the effectors which are responsible for activating cellular responses (Kandel et al., 2000). Cell signalling has been shown to be a dynamic process consisting of "on" mechanisms during which information flows down the pathway, and this is opposed by the "off" mechanisms that switch off the different steps of the signalling pathway (Berridge, 2006). The cell signalling model involves the effect of a stimulus on the receptors which lead to the activation of transducers and amplifiers that result in the generation of secondary messengers. The secondary messengers result in the modulation of the sensors and effectors which give rise to the cellular responses and effects such as cell growth and division, proliferation, differentiation or cell death. These cellular modulated activities are patterned and structured in a particular direction known as a signalling pathway which can be controlled by a series of substances, molecules and stimuli. As such, each of these pathways could be regulated, monitored, controlled, inhibited and exaggerated by some molecules and stimuli hence resulting in either increased or decreased output of the cellular activities (Kandel et al., 2000; Berridge, 2006).(Figure 1.1),

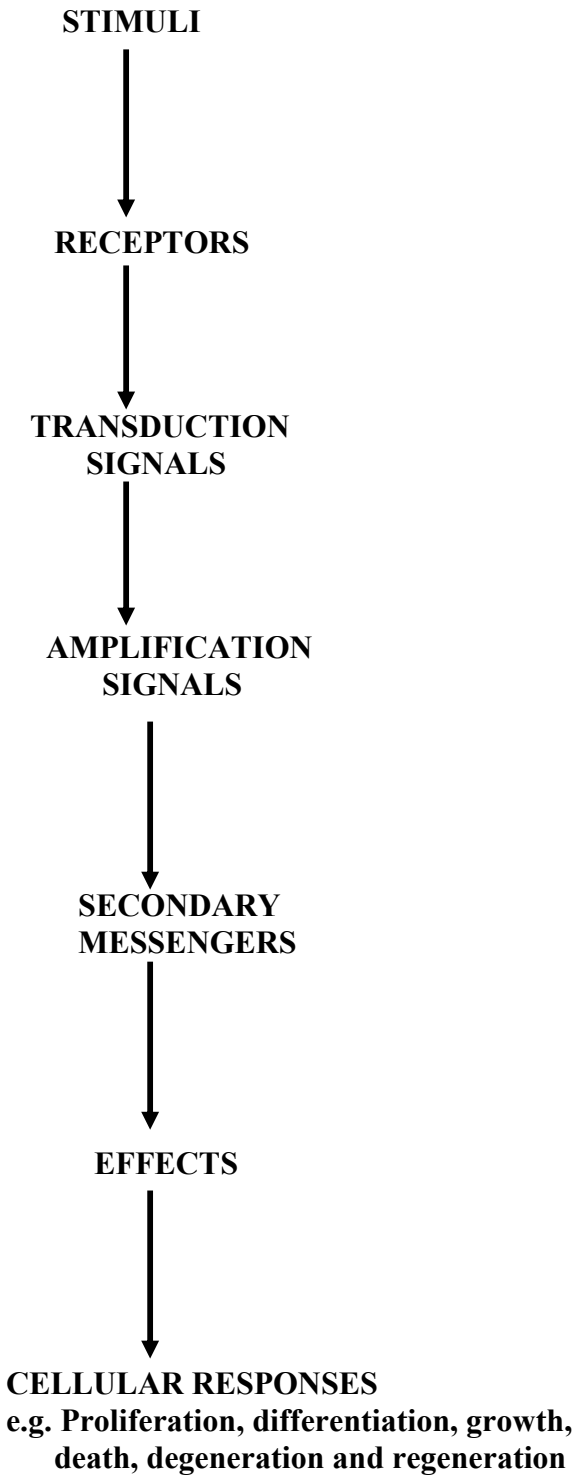


Fig.1.1. Schematic representation of a cell signalling pathway adapted from Berridge, (2006).

1.2.1. Neuronal cell signalling

The nervous system receives and transfers information, or signals, from the PNS to the CNS, processes the information in the CNS, and sends information back to the PNS. This transfer of information from the external environment, through neurons, and back again to the external environment is known as neuronal signalling. Neurons receive signals in two different forms: through chemical changes via neurotransmitters or receptors; and physical changes through touch receptors and photoreceptors. These signals cause ionic fluctuations in the plasma membrane, which in turn cause depolarization and generate electrical current flow in the neuron causing action potential generation which then travels along the neural membrane (Mel and Schiller, 2004).

The electrical nature of neuronal signalling showed that the output of most neurons is a pattern of spikes of action potentials, where the inside of the neuronal membrane is normally electrically negative and the action potential is a transient depolarisation of the cell membrane (Mel and Schiller, 2004). It has been shown that diseases that affect action potential generation and propagation also affect neuronal signalling (Croxford, 2003). Mel and Schiller (2004) have shown that the neuronal activities depend on how frequent the excitatory and inhibitory inputs are. The impact on the postsynaptic cell depends not only on the number and strength of excitatory and inhibitory synapses, but also on their location relative to one another. The activity of the postsynaptic neuron will influence the activity rates of downstream neurons (Gough et al., 2005). The balance of excitation and inhibition is crucial to overall neuronal and brain function. If the balance of the activity is too far towards excitation, it will result in over excitation leading to seizure, while if a shift is towards inhibition, it can lead to a dormant state; hence this condition may predispose the brain to pathophysiological conditions (Adler and Gough, 2005). Adler (2006a) have shown that neuronal activity leads to persistent increase in the efficacy of excitatory synapses in the brain resulting in long-term potentiation (LTP) which is involved in memory storage. Memory and even LTP have been shown to be an adaptive phenomenon that depends on neuronal activity. Signalling through ionotropic and metabotropic glutamate receptors has been implicated in central mechanisms involved in the pathogenesis of neuropathic pain, which is also associated with abnormal neuronal activity (Adler, 2006a).

Some signalling transduction pathways respond differently depending on the type and amount of signalling received by the cell (Chen et al., 2005a). Hence complex multi-component signal transduction pathways provide opportunities for feedback, signal amplification, and interactions inside one cell between multiple signals and signalling pathways (Adler, 2006a). The calcium ion (Ca^{2+}) is a highly versatile intracellular signal that operates to regulate many different cellular processes, and many of the Ca^{2+} signalling components are organized into molecular complexes in which Ca^{2+} signalling functions are carried out in localized areas (Berridge et al., 2003). These complexes can operate as autonomous units that can be multiplied or mixed and matched to create larger, more diverse signalling systems, as in the case of cardiac Ca^{2+} signalling. Many disease conditions such as hypertension, heart disease, diabetes, manic depression and Alzheimer's disease may occur as a result of abnormal remodelling of Ca^{2+} signalling (Berridge et al., 2003). Intercellular communication in multicellular organisms requires the relay of extracellular signals by cell surface proteins to the interior of cell as shown in Figure 1.2.

The signalling mechanisms that regulate actin-based motility processes in the nervous system have been shown by Meyer and Feldman (2002). They showed that both the migration of neurons and the extension of neurites require organised actin polymerization to push the cell membrane forward. Numerous extracellular stimulants of motility and axon guidance signals regulate actin-based motility through the rho GTPases and hence actin-based motility is critical for nervous system development. The signalling mechanisms by which extracellular signals guide axons to their targets lead to direct effects on actin filament dynamics (Mayer and Feldman, 2002) and, as such, showed an organised pattern which result in normal neuron development. Communication between neurons in the central nervous system occurs at specialized synaptic contacts and the strength of a synaptic signal is precisely regulated. The modification of synaptic strength is important in complex brain functions such as learning, memory and in the formation of appropriate neural maps during development (Walmsley, 1998). It has been shown that the fundamental building block of synaptic signalling is that the post-synaptic current is generated in response to the pre-synaptic release of a group of neurotransmitters (Walmsley, 1998). This shows that the neurotransmitter input from the pre-synaptic area determines the signalling output from the post-synaptic area. Hence if the input from the pre-synaptic

area is strong, the current generated at the post-synaptic area would be strong in response to the nature of the input. Dysregulation of this programme is thought to contribute to neurodegenerative diseases which are characterized by loss of the neurons (Gotz 2000).

Glial cells are active partners of neurons in processing information and synaptic integration and they receive coded signals from synapses and elaborate modulatory responses (Bezzi and Volterra, 2001). The active properties of glia, including long-range signalling and regulated transmitter release, and recent insights suggest that the active brain should no longer be regarded as a circuitry of neuronal contacts, but as an integrated network of interactive neurons and glia (Bezzi and Volterra, 2001). Within the developing nervous system, astrocytes and Schwann cells actively help to promote synapse formation and function, while in the adult brain, astrocytes respond to synaptic activity by releasing transmitters that modulate synaptic activity and as such, glia have been shown to be active participants in brain function (Bezzi and Volterra, 2001).

1.2.2. Mechanisms of signalling

Most cells possess receptors that operate through distinct effector mechanisms and the signalling pathways do not operate in isolation but may regulate and be regulated by each other called heterologous regulation (Golla and Seethala, 2002). In this way, the output of the cell is fine-tuned via subtle modulation of the relevant intracellular signalling mechanisms (Felipo, 2006). There are numerous examples in which the activity of one receptor can regulate, either positively or negatively, the activity of a second, e.g. increases in cAMP mediated by protein kinase A (PKA) can depress or potentiate phosphatidylinositol-specific phospholipase C (PI-PLC) activity (Golla and Seethala, 2002). Activation of PI-PLC, can result in modulation of the activity of other pathways via the activation of PKC. Also the same ligand may activate multiple pathways in a given tissue. For example, NE can activate β2-adrenergic receptors, which increase adenylyl cyclase; α2-adrenergic receptors are coupled to inhibition of adenylyl cyclase; and α1-adrenergic receptors are linked to the activation of PI-PLC (Rodrigo and Felipo, 2006). Similarly, Ach can activate muscarinic cholinergic receptors, which inhibit adenylyl cyclase, activate PI-PLC or activate K^+ channels. In

addition, ACh can directly activate nicotinic acetylcholine receptors (nAChRs), which are linked to changes in Na^+ and Ca^{2+} permeability (Rodrigo et al., 2007).

Thus, although individual signalling mechanisms are most frequently studied in isolation, their activity *in vivo* is likely to be highly regulated by other signal-transduction events (Golla and Seethala, 2002). A further consideration is that receptors which primarily activate one pathway may activate a second pathway. One example is the ability of GPCRs such as α2-adrenergic receptors or muscarinic acetylcholine receptors (mAChRs), to activate the MAPK cascade (Golla and Seethala, 2002). Activation of AC due to the stimulation of GPCRs, results, in the release of G protein βγ subunits, via an intermediary protein tyrosine kinase (PTK-X), which stimulates the phosphorylation of the adaptor protein Shc in the human granulosa cells (Asboth et al., 2001). The phosphorylation of the adaptor proteins then recruits the growth factor- binding protein 2/son of sevenless (Grb2-SOS) complex which then activates the MAPK pathway (Felipo, 2006; Willoughby and Cooper 2007). The activation of PI-PLC-linked receptors such as the mAChR results in increased PKC activity and since the addition of phorbol esters, which are PKC agonists, results in phosphorylation of Raf, this mechanism may provide an explanation for the ability of PI-PLC-coupled receptors to activate MAPK (Rodrigo et al., 2007). The effect as seen in granulosa cells may be different to other cells because as Asboth et al.(2001) have shown there are at least nine mammalian adenylyl cyclase isoenzymes, which may show different sensitivities towards other signalling systems in different cells.

Signalling molecules have been shown to activate gene transcription and in addition to their ability to elicit acute effects within cells, second-messenger molecules, such as cAMP, Ca^{2+} and DAG, can regulate gene transcription (Rodrigo et al., 2005). The transcription factors, cAMP response element-binding (CREB) protein, fos and jun, which respond to these signalling molecules, are members of the amphipathic helix family of proteins, and each contains a characteristic leucine zipper, which mediates dimerization (Golla and Seethala, 2002). These effects are possible because cells do not act in isolation, a signalling event in one cell may have a significant impact on the activity of neighbouring cells. Initial evidence for intracellular, nonsynaptic signalling was obtained from experiments with blood vessels in which it was observed that

addition of ACh increased cGMP concentrations and resulted in vasodilation and relaxation of the vascular smooth muscles (Golla and Seethala, 2002).

Activation of the MAPK pathway leads to increased expression of fos and jun. As increases in mRNA for fos and jun are observed very rapidly following a variety of stimuli, they have been termed immediate early genes and CREB has been implicated in long-term potentiation (LTP) and memory (Felipo, 2006).

1.2.3. Influence of membrane lipid on signalling

Plasma membrane lipids not only serve as an inert support for membrane proteins but also play an active role in the activity of a cell that is yet to be fully understood (Yang et al., 2005a). Membrane exocytic and endocytic processes, diffusion of macro-molecules and protein activities, among other cellular events, depend on the physical properties of membrane lipids. Vogler et al. (2004) have demonstrated that the hexagonal phase propensity of membranes differentially influences the binding of $G\alpha_i$, $G\beta\gamma$ and $G\alpha\beta\gamma$ proteins, indicating that membrane composition and structure can regulate cell signalling.

It has been shown that not only G protein localization is regulated by the membrane structure and composition, but also the function of these transducers and related proteins (Yang et al., 2005a). The type and abundance of membrane lipid species is regulated by dietary fat intake, which thus influences the properties of the membrane (Escudero et al., 1998;Yang et al., 2005a). Yang et al. (2005a) have demonstrated that the antitumor drug daunorubicin and oleic acid changed the membrane structure and inhibited G protein activity in biological membranes. These fatty acids are chemical but not structural analogues of oleic acid, supporting the structural basis of modulation of membrane lipid organization and subsequent regulation of G protein-coupled receptor signalling (Yang et al., 2005a). Yang et al. (2005a) investigated GPCR-mediated signalling to determine the effect of fatty acids on the membrane lipid structure and propagation of signals through these receptors. They showed that lipid structure regulates the interaction with membranes, recruitment to membranes and distribution to membrane domains of heterotrimeric $G\alpha\beta\gamma$ proteins, $G\alpha$ subunits and $G\beta\gamma$ dimers.

Moffet et al. (2000) have shown the importance of membrane lipid and protein-lipid interactions in that the initial steps of signalling cascades are associated with membranes and with the propagation of signals across these barriers. The signalling pathways are more dependent on the initial membrane-associated signalling elements; the receptors, G proteins and effectors than on the downstream signalling proteins that simply amplify the signals received. The receptors, G proteins and effectors can be recruited to certain specific membrane structures like membrane rafts (Moffet et al., 2000), which define their activities.

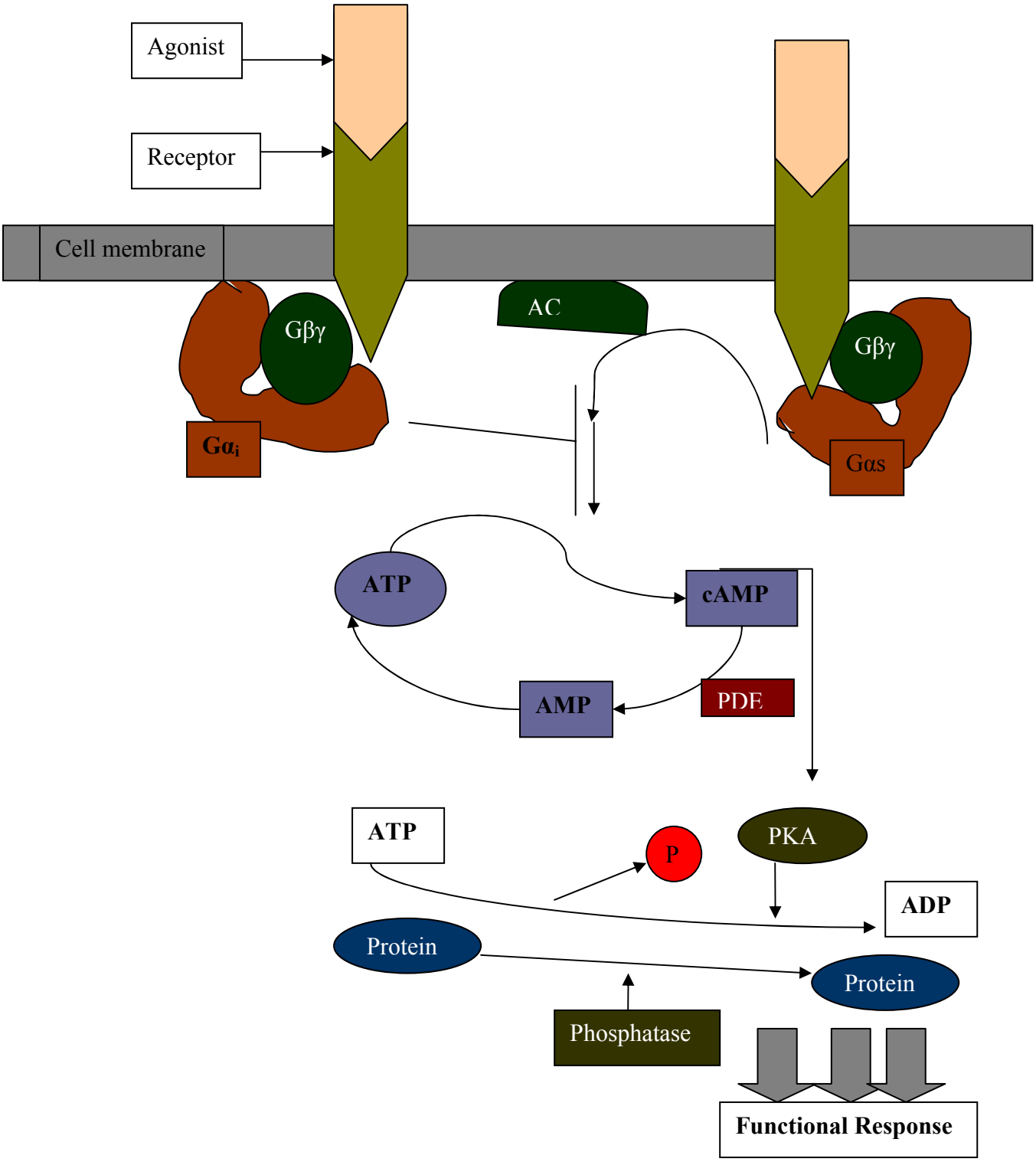


Fig.1.2. Schematic representation of the effects of agonist-induced activation of receptors at the cell membrane adapted from Sigma, (2006).

1.3. Receptors

Receptors are proteins that are found on the cell membrane, cytoplasm or nuclear membrane that bind specific molecules such as neurotransmitters, hormones or other ligands and as such initiate the cellular response to these substances (Ben-Shilomo et al., 2003). Ligand-induced changes in the behaviour of receptor proteins result in physiological changes that constitute the biological actions of the ligands. There are a variety of different types of ligands. Full agonists are able to activate the receptors and produce a maximal biological response and, as such, most naturally occurring ligands are full agonists. Partial agonists are not able to maximally activate receptors resulting in a partial biological response while antagonists bind to the receptor but do not activate it and thus result in receptor inhibition of the binding of agonists. Inverse agonists are ligands which produce an effect opposite to that of an agonist, yet act at the same receptor. They promote or stabilize an inactive form of the receptor (Howlett, 2005).

Receptors are activated by the binding of the specific agonist to them, which in turn leads to the activation of the second messenger. The activation of the second messenger cascade and the final biological response is achieved only when a significant number of receptors are activated by bound ligands. There are different types of receptors which depend on the ligand, function and position (Ben-Shilomo et al., 2003). Some receptor proteins are peripheral membrane proteins while many hormone receptors and neurotransmitter receptors are transmembrane proteins.

The transmembrane receptors are embedded in the lipid bilayer of cell membranes and allow the activation of signal transduction pathways in response to the activation by the binding molecule, or ligand (Berridge, 2006). Metabotropic receptors are coupled to G proteins and affect cells indirectly through enzymes which control ion channels, while ionotropic receptors contain a central pore which functions as a ligand-gated ion channel (Howlett, 2005). Another major class of receptors are intracellular proteins, such as steroid hormone receptors. These receptors can often enter the cell nucleus and modulate gene expression in response to the activation by the ligand (Connor and Christie, 1999; Garzon et al., 2005). The regulatory protein subunits of many ion channels and transmembrane receptors, for example, may be defined as peripheral membrane proteins (Connor and Christie, 1999).

The transmembrane receptors are integral membrane proteins, which reside and operate in the plasma membrane of the cell and also in the membranes of some subcellular compartments and organelles (Chow and Stahelin, 2005). The binding of signalling molecule on one side of the membrane to transmembrane receptors initiate a response on the other side. In this way they play a unique role in cellular communications and signal transduction (Berridge, 2006). Many transmembrane receptors are composed of two or more protein subunits which operate collectively and may dissociate when ligands bind or fall off. The transmembrane receptors have both extracellular domain and intracellular domain which means that within the intracellular domain enzymes are found that bring about the phosphorylation of the effectors (Berridge et al., 2003).

There are several ways that cells regulate the activity of a transmembrane receptor. The most important way of regulation is through phosphorylation and internalization (Kandel et al., 2000). Examples of transmembrane receptors are adrenergic receptors, olfactory receptors, receptor tyrosine kinases, epidermal growth factor receptors, insulin receptors, fibroblast growth factor receptors, neurotrophin receptors, nerve growth factor (NGF) receptors and N-methyl-D-aspartic acid (NMDA) receptors (Purves et al., 2001).

Neurotransmitter receptors can be classified into two broad categories based on their structural and functional characteristics, namely metabotropic and ionotropic receptors. The metabotropic receptors do not form an ion channel pore, rather, they are indirectly linked with ion-channels on the plasma membrane of the cell through signal transduction mechanisms (Berridge, 2006). This class of receptor includes the metabotropic glutamate receptors, muscarinic acetylcholine receptors, γ-amino butyric acid (GABA) receptors, and most serotonin receptors, as well as receptors for norepinephrine, epinephrine, histamine, dopamine, endorphin, enkephalin and the endocannabinoids (Purves et al., 2001).

All metabotropic receptors are monomeric proteins with seven transmembrane domains. The N terminus of the protein is on the extracellular side of the membrane and its C terminus is on the intracellular side (Purves et al., 2001). Metabotropic receptors have neurotransmitters as ligands, which, when bound to the receptors,

initiate a cascade of events that can lead to channel-opening or other cellular effects (Berridge, 2006). When a ligand or primary messenger, binds to the receptor, or the transducer, it activates a primary effector, which can go on to activate secondary messengers. Since the opening of channels by metabotropic receptors involves activating a number of molecules in turn, channels associated with these receptors take longer to open than ionotropic receptors do, and are thus not involved in mechanisms that require quick responses (Kandel et al., 2000). Metabotropic receptors also remain open from seconds to minutes and have a much longer-lasting effect than ionotropic receptors, which open quickly but only remain open for a few milliseconds (Kandel et al., 2000; Berridge et al., 2003). While ionotropic channels have an effect only in the immediate region of the receptor, the effects of metabotropic receptors can be more widespread through the cell (Berridge, 2006).

Metabotropic receptors can open and close channels and can make a membrane more excitable by closing K^+ channels. They can retain positive charge within the cell and thus reduce the amount of current necessary to cause an action potential (Kandel et al., 2000). The metabotropic receptors on the presynaptic membrane can inhibit or, more rarely, facilitate neurotransmitter release from the presynaptic neuron (Schmitz et al., 2001). These receptors can be further classified into receptor tyrosine kinases and G-protein-coupled receptors, known as GPCRs (Kandel et al., 2000).

GPCRs are also known as seven transmembrane (7TM) receptors. Some of the examples are muscarinic acetylcholine receptors, adenosine receptors, adrenergic receptors, γ-amino butyric acid (GABA), cannabinoid receptors, dopamine receptors, glucagon receptors, metabotropic glutamate receptors, opioid receptors and calcium-sensing receptor and olfactory receptors. Hence the majority of life processes occurs through the activation of the GPCRs. Cells can regulate their receptors by either downregulating or upregulating the number of receptors to a given hormone or neurotransmitter in order to alter their sensitivity to that molecule. This is called local feedback mechanism (Berridge, 2006; Kandel et al., 2000).

1.3.1 G proteins

Guanine nucleotide binding proteins (G-proteins) are important mediators of cellular functions. They are characterised by a recognition site for guanine nucleotides namely

guanine triphosphate (GTP) and guanine diphosphate (GDP), and possess intrinsic GTPase activity (Siegel et al., 1999). The G proteins play a central role in signal transduction and many other cellular processes. They are divided into three distinct groups namely, the switches, the sensors and the clocks (Siegel et al., 1999).

The switches are the small G proteins that play important roles in cell function such as cytoskeletal remodelling, cellular differentiation and vesicular transport. The small G proteins, like other G proteins, bind guanine nucleotides, possess intrinsic GTPase activity and cycle through GDP-and GTP-bound forms (Siegel et al., 1999; Blaukat et al., 2000). The small G proteins function as molecular switches that control several cellular processes and examples include ras, rap, ran and ADP-ribosylation factor 1, as shown in Table 1.1.

The ras p21 protein plays an important role in the regulation of cell differentiation through the stimulation of receptor tyrosine kinases (Blaukat et al., 2000). The binding of a growth factor to a receptor, stimulates the autophosphorylation of tyrosine kinase which assists in the recruitment of exchange factors. These exchange factors stimulate GDP-GTP exchange on the small monomeric G protein ras, leading to the activation of mitogen-activated protein kinases (MAPKs). MAPKs are involved in the phosphorylation of transcription factors that stimulate gene transcription (Siegel et al., 1999; Dhanasekaran and Prasad 1998; Cabrera-Vera et al., 2003).

Table 1.1 Examples of small G proteins and their cellular functions (Siegel et al.,1999).

Class	Cellular function
Ras	Signal transduction (control of growth factor and MAP-kinase pathways)
Rac, CDC42	Signal transduction (control of cellular stress responses and MAP-kinase*** pathways)
Rab	Localized to synaptic vesicles, where it regulates vesicle trafficking and exocytosis.
Rho	Assembly of cytoskeletal structures (e.g., actin microfilaments)
ARF*	ADP-ribosylation of Gαs; Assembly and function of Golgi complex.
EFTU**	Association with ribosomes, where it regulates protein synthesis.
Ran	Nuclear-cytoplasmic trafficking of RNA and protein

ARF*: ADP-ribosylation factor; EFTU**: eukaryotic elongation factor; MAP-kinase***: Mitogen activated protein kinase.

The sensors are the translation and elongation factors such as Tu and G. The translation factors play a pivotal role in protein synthesis especially in the second step of the three-step translation process (initiation, elongation and termination) (Cabrera-Vera et al., 2003). These GTP-binding elongation factors are responsible for two elements of elongation. The elongation factor Tu escorts the tRNA carrying the correct amino acid to the correct site on the mRNA, where GTP-GDP exchange takes place. The elongation factor G is involved in the translocation of tRNA from the aminoacyl site to the peptidyl site on the ribosome which also involves GTP hydrolysis (Siegel et al., 1999). The clocks are the heterotrimeric G proteins present within the cytoplasm and are linked with GPCRs in the cell membrane as discussed in section 1.3.2. G protein activation occurs as shown in Figure 1.3.

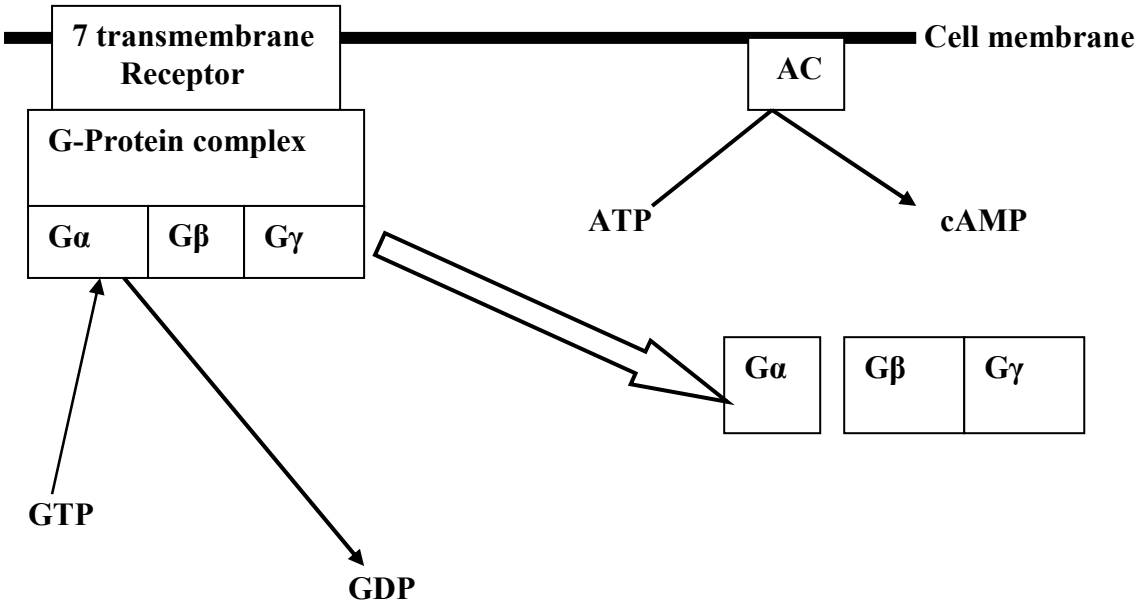


Fig.1.3. Activation of G protein by agonists result in the use of GTP to release GDP and AC phosphorylating ATP to yield cAMP. Adapted from Cabrera-Vera et al.(2003).

1.3.2. Heterotrimeric G proteins

The heterotrimeric G proteins have been shown to be important in signal transduction and are located at the cytoplasmic face of the plasma membrane, where they interact with the membrane–spanning GPCRs and effector molecules (Siegel et al., 2006). G protein heterotrimers consist of Gα, Gβ & Gγ subunits. Lipid modification of Gα and Gγ subunits, help to anchor the G protein heterotrimer to the plasma membrane (Cabrera-Vera et al., 2003). The Gα subunits bind guanine nucleotides with high affinity and contain an intrinsic GTPase activity (Siegel et al., 1999). The ability of Gα subunits to bind guanine nucleotides arises from their homology with other members of the GTP binding protein super family, including small proteins such as p21, Ras, Rab, ran, Ral, rac, Rho, and EF-Tu (Siegel et al., 1999; Cabrera-Vera et al., 2003; Fromm et al., 1997; Oldham and Hamm, 2006).

Gβ & Gγ subunits form a very tight, noncovalent heterodimer and function as a single entity (the Gβγ complex) throughout the G protein signalling cycle (Dolphin,1996). Each heterotrimeric G protein has been shown to have different subunits and the combination of these subunits define the specific role of each G protein, however not all combinations are functional (Siegel et al., 1999). The G protein α subunit contains a binding site for a guanine nucleotide, which allows the binding of GDP in its non-activated state (Siegel et al., 2006). The G protein activation results in the exchange of GDP for GTP, on the Gα subunit. The Gα subunit is therefore activated, facilitating its dissociation from the Gβ and γ subunits respectively (Dolphin 1996; Durchánková et al., 2008). These activated Gα subunits then regulate the activity of effector enzymes such as phospholipase C, phospholipase A_2, and ion channels like K^+ or Ca^2 (Flavahan & Vanhoutte,1990; Durchánková et al., 2008).

Although the Gα subunit interacts with different effector domains according to each G protein, the Gβ and Gγ subunits appear to be interchangeable (Siegel et al, 1999). Other G proteins have distinct Gβ and Gγ subunit differences and these subunits may play a role in signal production and transduction (Levitzki,1990; Wang, 1999; Zhong, 2003; Waters et al., 2004). The dissociation of Gα subunit and the effector is regulated by the intrinsic GTPase activity of the Gα subunit (Sprang, 1997). G proteins may be activated many more times before desensitisation of the receptor and consequently the reassociation of the G protein components together (Levitzki,1990;

Siegel et al., 1999; Durchánková et al., 2008) as shown in Figure 1.4.

The different types of G protein contain distinct α subtypes, which in part, confer the specificity of functional activity. The types of G protein α subunit are categorized based on their structural and functional homologies (Siegel et al.,1999; Sprang, 1997; Durchánková et al., 2008). The molecular weight (Mr) of these proteins varies between 38,000-52,000. Multiple subtypes of β and γ subunits include five β subunits of Mr 35,000–36,000 and seven γ subunits of Mr 6,000-9,000. These proteins show distinct cellular distributions and differences in their functional properties (Siegel et al., 2006; Morris & Malbon, 2000; Oldham and Hamm, 2006). Multiple forms of heterotrimeric G proteins have been shown to exist in the nervous system (Siegel et al., 1999; Oldham and Hamm, 2006). Three types of heterotrimeric G protein have been identified according to Siegel et al, (1999). G_t, or transducin, was identified as the G protein that couples rhodopsin to regulate photoreceptor cell function, and G_s and G_i were identified as G proteins that couple plasma membrane receptors to the stimulation and inhibition, respectively, of adenylyl cyclase, the enzyme that catalyzes the synthesis of cAMP (Siegel et al.,1999; Benians et al., 2005). Since the early 1990s, over 35 heterotrimeric G protein subunits have been identified by a combination of biochemical and molecular cloning techniques (Siegel et al., 2006; Mullaney, 1999; Oldham and Hamm, 2006; Dignard et al., 2008). In addition to G_t, G_s and G_i, the other types of G protein in the brain are designated as G_o, G_{olf}, G_{gust}, G_z, G_q and $G_{11\text{-}16}$. Moreover, for some of these G proteins, multiple subtypes show unique distributions in the brain and peripheral tissues (Mullaney,1999; Siegel et al., 2006; Neer,1995; Kitanaka et al., 2008), as shown in Table 1.2. The activation of G protein is shown in Figure 1.4.

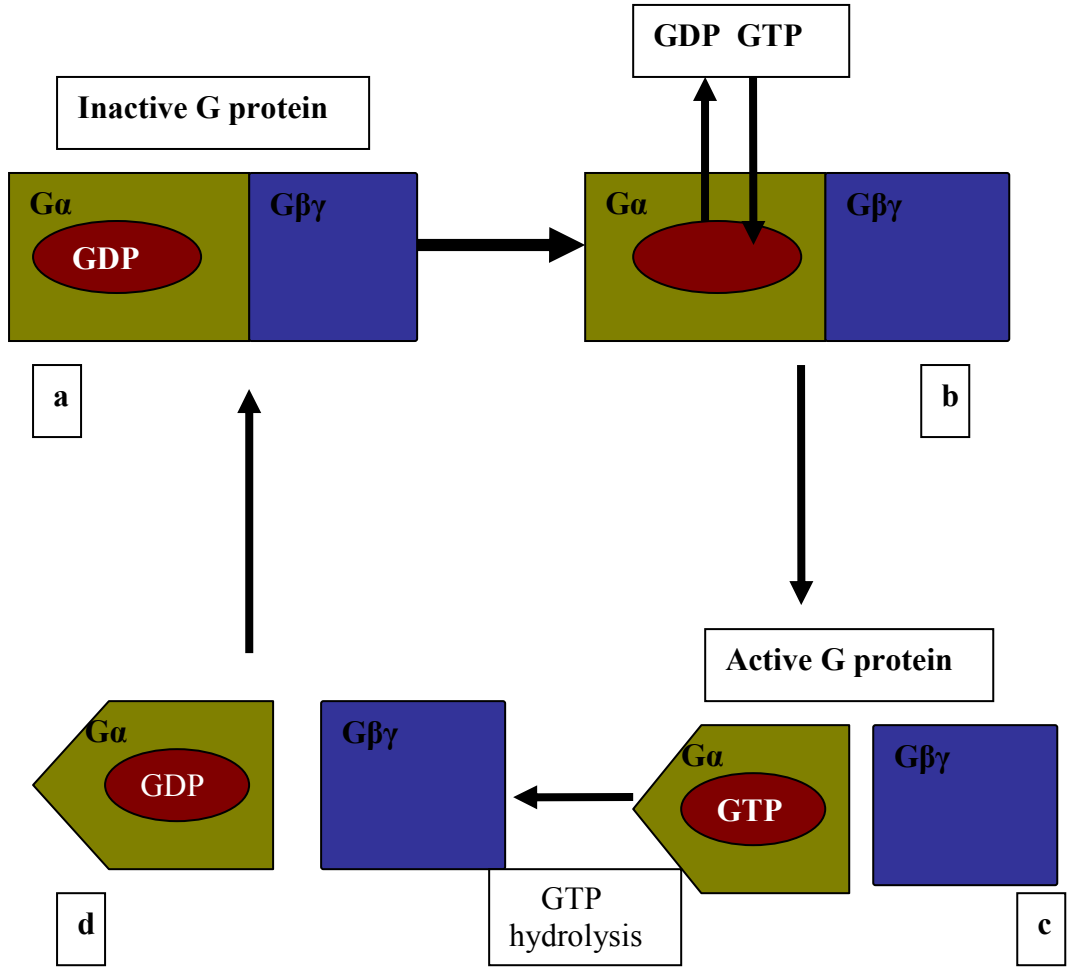


Fig.1.4: G protein cycles between active and inactive states involving
a) Gα binds to guanosine diphosphate (GDP) in inactive state.
b) Stimulation of the receptor by the agonist, leads to the release of the GDP, of which the GTP then binds to the empty site because its concentration is higher than the GDP.
c) The dissociation of βγ subunits due to low affinity of GTP-bound Gα
d) GTP is hydrolysed to GDP due to GTPase activity of Gα.

Table 1.2. Heterotrimeric G protein α-subunits in brain adapted from Siegel et al., 1999.

Family	Molecular Weight (Mr)	Effector protein(s)
Gs		
G αs_1	52,000	Adenylyl cyclase (activation)
$G\alpha s_2$	52,000	
$G\alpha s_3$	45,000	
$G\alpha_4$	45,000	
Gαolf	45,000	
Gi		
$G\alpha i_1$	41,000	Adenylyl cyclase (inhibition)
$G\alpha i_2$	40,000	K+ channel (activation)
$G\alpha i_3$	41,000	Ca2+ (inhibition) PI-Phospholipase C (activation) Phospholipase A_2
$G\alpha o_1$	39,000	K+ channel (activation)
$G\alpha o_2$	39,000	Ca2+ channel (inhibition)
$G\alpha t_1$	39,000	Phosphodiesterase (Activation) in rods and cones.
$G\alpha t_2$	40,000	
Gαgust	41,000	Phosphodiesterase (activation) in taste epithelium
Gαz	41,000	Adenylyl cyclase (inhibition)
Gq	41,000-43,000	
Gαq		PI-Phospholipase C (activation)
$G\alpha_{11}$		
$G\alpha_{14}$		
$G\alpha_{15}$		
$G\alpha_{16}$		
G_{12}	44,000	Unknown
$G\alpha_{12}$		
$G\alpha_{13}$		

1.3.3. The Gα subunit

The Gα subunits are divided into four classes ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$), based on their respective amino acid sequences (Table 1.2). Each of these classes has at least two subtypes. The $G\alpha_s$ class includes subtypes $G\alpha_s$ and $G\alpha_{olf}$. $G\alpha_{olf}$ is located in chemosensory neurons only (Novotny & Svoboda, 1998; Kitanaka et al., 2008). $G\alpha_s$ stimulates adenylate cyclase (AC)1-6, thereby increasing cAMP, leading to the phosphorylation and subsequent activation of Ca^{2+} channels (Dolphin,1996). This G protein is also associated with the inactivation of cardiac Na^+ channels and may be directly coupled to intracellular Ca^{2+} channels (Novotny & Svoboda, 1998; Dolphin, 1996; Straiker et al., 2002). ADP-ribosylation of the $G\alpha_s$ subunit which is catalysed by cholera toxin, causes an increase in AC by slowing the 'off' phase of GTPase reaction (Levitzki, 1990; Oldham and Hamm, 2006).

Two variants of $G\alpha_s$ have been shown in both humans and animals- the short (~44kDa) and the long (~46kDa) (Milligan et al.,1999; Oldham and Hamm, 2006). The majority of $G\alpha_s$ variants located in the kidney, placenta, cortex, cerebellum and adrenal medulla are $G\alpha_{s\text{-}L}$, however $G\alpha_{s\text{-}S}$ predominates in the heart, liver, neostriatum and platelets (Novotny & Svoboda, 1998). Both variants of these G proteins are functionally similar, however a measurable difference in the rate of GDP dissociation is observed. $G\alpha_{s\text{-}S}$ may have a higher efficacy in stimulating AC in some cells and the cellular distribution of the two variants also varies (Novotny & Svoboda, 1998; Durchánková et al., 2008). $G\alpha_i$ class includes $G\alpha_{i,}$ $G\alpha_t$, $G\alpha_o$ $G\alpha_{gust}$ and $G\alpha_z$. $G\alpha_i$ inhibits AC, decreases cAMP and activates K^+ channels. The $G\alpha_o$ (molecular weight 39kDa), inhibits Ca^{2+} channels, is present in brain tissues and, is believed to be involved in neuronal responses and has a (Milligan, et al., 1990, Kaziro, et al., 1991; Hepler & Gilman, 1992; Durchánková et al., 2008). The $G\alpha_q$ activation stimulates PLC-β leading to increased Ca^{2+} and subsequent activation of PKC which respectively activates AC1 and AC2 leading to increased cAMP production and finally the activation of PKA (Dolphin, 1996; Murray and Shewan, 2008). The Gα subunits are shown in Figure 1.5.

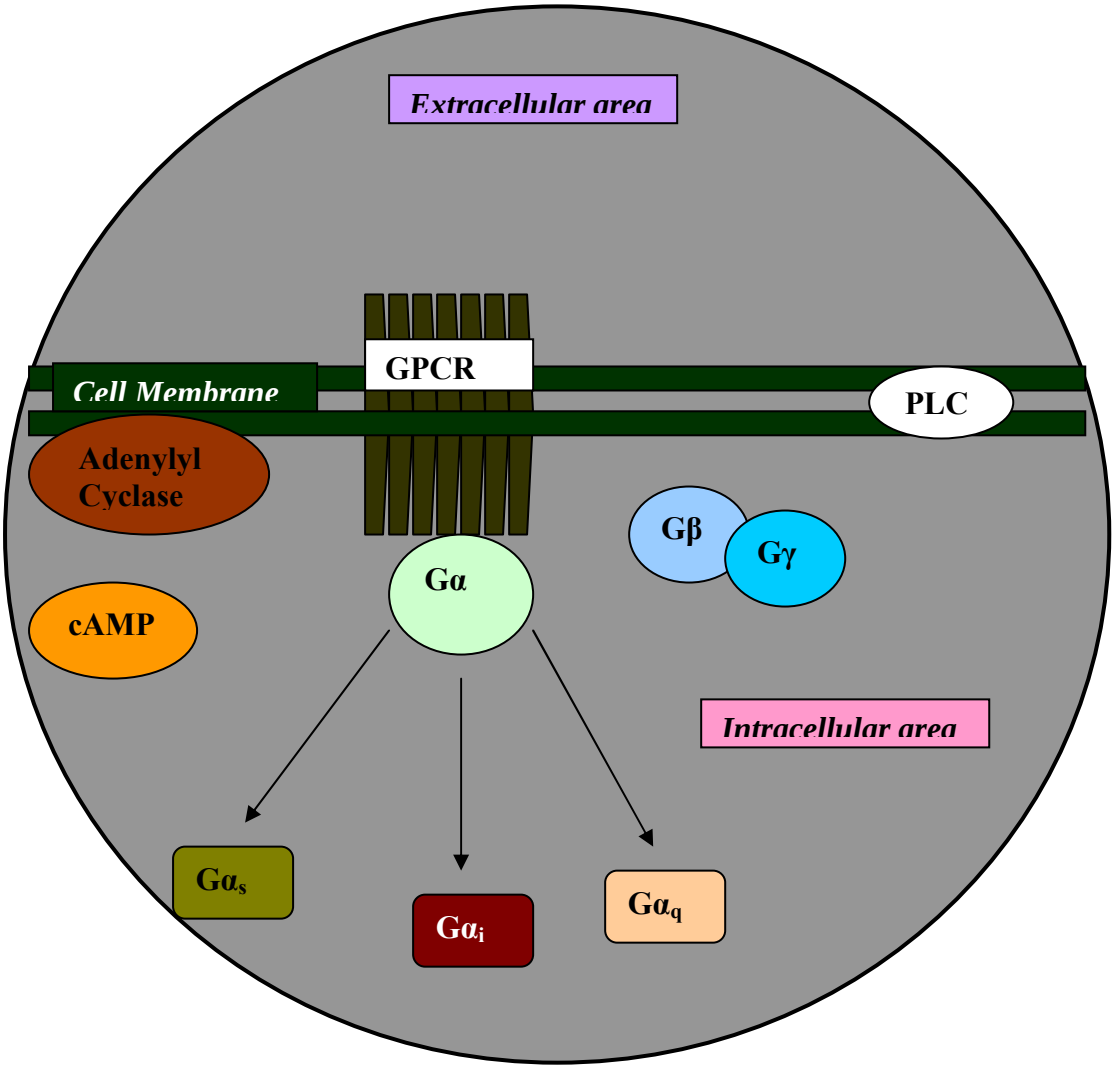


Fig.1.5.The G protein alpha subunits activation through G protein coupled receptors present on the cell membrane. Adapted from Sigma, (2006).

1.3.4. The Gβγ dimer

The tightly bound Gβγ dimer has been shown to regulate many effectors and may be involved in GPCR kinase recruitment and hence involved in the activation of second messengers (Hamm & Gilchrist,1996; Dignard et al., 2008). The Gβ subunit is approximately 36kDa and comprises subtypes that are highly homologous. The Gγ subtypes about 6-9kDa are more divergent and are thought to account for the functional differences in Gβγ (Hamm & Gilchrist,1996). The Gβγ dimer has been demonstrated to have several roles in signal transduction. The Gβγ subunits are

membrane associated, due to the isoprenylation of the Gγ subunit, which is also necessary for the effective interaction of Gβγ with Gα (Muller & Lohse, 1995).

When GTP is bound to the Gα subunit, the heterotrimeric complex becomes activated and the subsequent dissociation of Gα from Gβγ allows the Gβγ subunit to interact with effectors. Although little is known about the specific sites on the Gβ or Gγ subunit that interact with effector systems, it is now known that Gβγ dimer is important in effector activation (Hamm & Gilchrist,1996; Blackmer et al., 2001). Some forms of AC such as 1,2 and 4 are stimulated or inhibited by interactions with Gβγ (Muller & Lohse,1995). These subunits can also influence GRK transportation to the cell membrane, K^+ channel opening frequency and other effectors such as phospholipase A_2 (PLA_2). The Gβγ is also involved in the inhibition of unidentified Ca^{2+} currents, possibly by facilitating alterations in the closed state of the ion channel, making the ion channel less willing to open (Clapham, 1996; Blackmer et al., 2001).

1.3.5. The function of G protein βγ subunits

It has been demonstrated that one group of protein kinase, the G protein receptor kinases (GRKs), can bind to βγ subunits. These kinases phosphorylate G protein-coupled receptors that are occupied by ligand and thereby mediate one form of receptor desensitization (Siegel et al., 2006). It is now known that βγ play a very important role in receptor desensitisation and the GRK is normally a cytoplasmic protein that does not come in appreciable contact with the plasma membrane receptor under basal conditions (Siegel et al., 2006). Ligand binding to the receptor activates the associated G protein, which results in the generation of free α and βγ subunits. The βγ subunits, which remain membrane-bound, are now free to bind to the C-terminal domain of GRK. This draws the GRK into close physical proximity with the receptor and enables receptor phosphorylation. In this way, the βγ subunits target GRKs, which have constitutive catalytic activity to those receptors that are ligand-bound (Hamm & Gilchrist,1996; Siegel et al., 2006; Dignard et al., 2008).

Another important role of βγ subunits is the regulation of the mitogen-activated protein kinase (MAP-kinase) pathway (Siegel et al., 1999). MAP-kinases are the major effector pathway for growth factor receptors however, signals that act through GPCRs, particularly those coupled to G_i, can modulate growth factor activation of

MAP-kinase pathway. This is mediated via βγ subunits (Siegel et al., 2006). Activation of receptors leads to the generation of free βγ subunits which then activate the MAP-kinase pathway at some early step in the cascade (Siegel et al., 2006). Some possibilities are by direct action of the βγ subunits on Ras or on one of several linker proteins between the growth factor receptor itself and activation of Ras (Hamm & Gilchrist,1996; Siegel et al., 2006; Dignard et al., 2008).

1.3.6. Modulation of heterotrimeric G proteins

The functioning of heterotrimeric G proteins is modulated by several other proteins. One major class of modulator protein binds to G protein α subunits and stimulates their intrinsic GTPase activity. These are called GTPase-activating proteins (GAPs). The GAPs had been known to exist for small G proteins but have been identified for heterotrimeric G proteins (Siegel et al., 1999). The GAPs have been termed regulators of G protein-signalling (RGS) proteins. It has been shown that activation of α subunit, GTPase activity hastens the hydrolysis of GTP to GDP, more rapidly restores the inactive heterotrimer and, hence, the RGS proteins inhibit the biological activity of G proteins (Siegel et al., 1999). Nunn et al., (2006) and Siegel et al. (2006) have shown that some 20 forms of mammalian RGS protein are now known and most are expressed in the brain with highly region-specific patterns. It is also thought that different families of G protein α subunits are likely to be modulated by different forms of RGS protein (Siegel et al., 2006; Nunn et al., 2006).

Another protein modulator of G protein function is phosducin, a cytosolic protein enriched in the retina and pineal gland but also expressed in the brain and other tissues of humans and other mammals (Danner & Lohse, 1996; Schroder and Lohse, 1996). Phosducin binds to G protein βγ subunits with high affinity and this results in the prevention of βγ subunit reassociation with the α subunit. In this way, phosducin may sequester βγ subunits which initially may prolong the biological activity of the α subunit (Hamm & Gilchrist,1996), which eventually may inhibit G protein activity by preventing the direct biological effects of the βγ subunits as well as regeneration of the functional G protein heterotrimer (Siegel et al., 2006).

Jiang et al.(1998), have shown that G_o is the most abundant G protein in neurons, where it constitutes up to 2% of membrane protein, and is also expressed in endocrine cells and the heart. Jiang et al.(1998), showed that G_o is activated not only by the same class of seven-transmembrane receptors that activate the inhibitory G proteins, but also by at least two proteins that do not belong to the rhodopsin-like family of G protein-coupled receptors and the Alzheimer amyloid protein precursor protein responsible for familial forms of the disease.

All G protein α subunits have been shown to be modified in their N- terminal domains by palmitoylation or myristoylation (Dohlman and Thorner, 1997). These modifications may regulate the affinity of the α subunit for its βγ subunits and, thereby the likelihood of dissociation or reassociation of the heterotrimer. The modifications also may help determine whether the α subunit, released upon ligand-receptor interaction, remains associated with the plasma membrane or diffuses into the cytoplasm. This could have important consequences on the types of effector proteins regulated. G protein γ subunits are modified on their C- terminal cysteine residues by isoprenylation (Jiang et al.,1998). There is evidence that this modification plays a key role in anchoring the γ subunit and its associated β subunit to the plasma membrane (Cabrera-Vera, et al., 2003; Slessareva et al., 2003). The importance of this anchoring shows the ability of βγ- subunits to target GRKs to ligand-bound receptors depends on this membrane localization (Siegel et al., 1999; Cabrera-Vera, et al., 2003).

1.3.7. G protein and ion channels

Many G proteins are linked to fluctuations in intracellular ion concentrations, which is due to both direct activation of ion channels by G proteins and indirect second messenger-mediated responses (Berridge et al., 2003; Lowes et al., 2002). AC is stimulated by the activation of G_s which results in the elevation of intracellular cAMP levels. This increase in cAMP can directly open Ca^{2+} channels or, alternatively, can activate Ca^{2+} and K^+ channels via cAMP-dependent phosphorylation of the channel. Protein kinase C (PKC) is involved in the phosphorylation of several Ca^{2+} channels in various cell populations including neurons. PKC is also involved in the inhibition of other ion currents including K^+, Ca^{2+}-dependent K^+, and Na^+ channels. Other

second messenger components also influence ion channel activation and inhibition including phospholipase A_2 (PLA_2) and intracellular Ca^{2+} levels (Berridge et al., 2003; Berridge, 2006; Burgoyne, 2007).

G proteins are involved in direct activation and inhibition of several ion channels. The stimulation of Ca^{2+} current has been associated with the direct interaction with G proteins and similarly the receptor-mediated inhibition of Ca^{2+} channels is also linked to G proteins (Dolphin, 1990; Berridge et al., 2003). G proteins couple some neurotransmitter receptors directly to ion channels and one of the best examples of this mechanism in the brain is the coupling of many types of receptors including μ-opioid, α_2-adrenergic, D_2-dopaminergic, muscarinic cholinergic, 5HT1a-serotonergic and $GABA_B$ receptors, to the activation of an inward rectifying K^+ channel (GIRK) via pertussis toxin-sensitive mechanisms (Wickman & Clapham, 1995: Schneider et al., 1997).

It has been shown that binding of the G protein subunits to the Ca^{2+} channels, reduces their probability of opening in response to membrane depolarization. This mechanism is best seen in L-type Ca^{2+} channels, which are inhibited by the dihydropyridine antihypertensive drugs such as verapamil but may also operate for other types of voltage-gated Ca^{2+} channel (Berridge et al., 2003). Another example of direct regulation of ion channels by G proteins is the stimulation L-type Ca^{2+} channels by G_s. In this case, free α subunits appear to bind to the channel and increase their probability of opening in response to membrane depolarization (Wickman & Clapham, 1995; Berridge et al., 2003).

1.3.8 G proteins and MAPK

Several G protein-coupled receptors are capable of activating the MAPK pathway (Luttrell et al., 1997 ; Lowes et al., 2002). Research has shown the involvement of a ras-dependent mechanism and MAPK was found to induce mitogenesis in cultured fibroblasts after stimulation of GPCRs by naturally occurring phospholipids (Howe & Marshall, 1993). MAPKs are localised in both the cytoplasm and nucleus and are suspected to be involved in the phosphorylation of nuclear transcription factors which regulate gene transcription (Luttrell et al., 1997). Activation of PKC and phospholipase C beta (PLCβ) has also been linked to MAPK activation (Kolch et al.,

1993). Thus, G proteins are linked to pathways that influence not only membrane conductance but also cell proliferation and growth, implicating a possible role of G proteins in disease pathology (Luttrell et al., 1997; Berridge, 2006; Cabrera-Vera, et al., 2003).

1.4. G proteins in disease

G Protein-mediated signal transduction is important in the regulation of a cell's morphological and physiological response to external factors (Wettschureck and Offermanns, 2005; Ohkubo and Nakahata, 2007). G proteins have been demonstrated to be involved in disease pathology through several mechanisms (Ohkubo and Nakahata, 2007). Among them are the exotoxins such as cholera or pertussis toxins which interfere with the G protein activity. Other disease pathologies associated with abnormal mutations in G proteins can interfere with signal transduction pathways and disease pathogenesis may also involve signal transmission that is either excessive, by augmentation of G protein function, or insufficient, via inactivation of G proteins (Wettschureck and Offermanns, 2005).

Specific mutations may affect the ability of a G protein to hydrolyze GTP which may interfere with signal initiation, transmission and termination (Ohkubo and Nakahata, 2007). Other mutations alter levels of a specific G protein or produce unstable G proteins, leading to changes in the response to a stimulus. Mutations may also alter the rate of GDP release and GTP binding, resulting in modifications to downstream signalling (Farfel et al., 1999; Xie and Palmer., 2007).

It has been shown that the regulators of G protein signalling (RGS) proteins may play a role in disease pathology, since the RGS proteins have been found to reduce termination times by accelerating GTP hydrolysis, and are important in mediating slowing of heart rate, photon detection in the retina and the contraction of smooth muscle cells (Berman & Gilman, 1998). Mutations of these RGS proteins may play a role in prolonged stimulation of effectors associated with these proteins (Farfel, et al., 1999; Lorenz et al., 2007).

Genetic variations and defects can also cause the inactivation of G proteins. Pseudohypoparathyroidism type 1 is caused by the null response of cells to parathyroid hormone and other hormones that are mediated by G_s. This may be due to either decreased levels of the active $G\alpha_s$ subunit or the production of inactive $G\alpha_s$ subunits (Farfel et al., 1999). Pseudohypoparathyroidism type 1b may also be caused by a genetic defect in $G\alpha_s$ (Farfel et al., 1999: Lorenz et al., 2007).

1.5. Second Messengers

Second messengers are molecules that relay signals received at receptors on the cell surface, such as the arrival of protein hormones and growth factors to target molecules in the cytosol or nucleus. In addition to this function as relay molecules, second messengers serve to greatly amplify the strength of the signal and the binding of a ligand to a single receptor at the cell surface. As a result they may end up causing massive changes in the biochemical and physical activities within the cell through the activities of the second messengers which amplify the effect of the signal on the effectors (Berridge et al., 2003; Berridge, 2006).

Second messengers are intracellular molecules or ions that are regulated by extracellular signalling agents such as neurotransmitters, hormones and their ligands which are called first messengers. Second messengers typically operate by activation of protein kinases that phosphorylate target proteins, thereby altering the function of these proteins (Kandel et al., 2000). Such functional effects are subsequently reversed by protein phosphatase-mediated dephosphorylation and the second messengers modulate a wide range of rapid and long-term neuronal processes (Garzon et al., 2003). Examples of second messenger molecules are cAMP, Ca^{2+} and inositol triphosphate (IP_3) (Heldin, 2003).

1.5.1. Types of second messengers

There are three main types of secondary messenger molecules:

(1) Hydrophobic molecules like diacylglycerol, inositol trisphosphate (IP_3) and phosphatidylinositols. These are membrane-associated and diffuse from the plasma membrane into the juxtamembrane space where they can reach and regulate membrane-associated effector proteins (Vanhaesebroeck et al., 1997; Cohen, 2002).

(2) Hydrophilic molecules are water-soluble molecules, like cAMP, cGMP, and Ca^{2+},

that are located within the cytosol and (3) gases, nitric oxide (NO) and carbon monoxide (CO), that can diffuse through cytosol and across cellular membranes (Chow and Stahelin, 2005). Secondary messengers can be synthesized or released and broken down again in specific reactions by enzymes. Some, like Ca^{2+}, can be stored in special organelles and quickly released when needed and their production, release and destruction can be localized, enabling the cell to limit space and time of signal activity (Siegel et al., 1999; Haglund and Dikic, 2003).

1.5.2. Mechanisms of action of secondary messengers

Cyclic AMP is synthesized from ATP by the action of the enzyme adenylyl cyclase (AC) and the binding of the hormone to its receptor activates a G protein which, in turn, activates AC (Vanhaesebroeck et al., 1997). The resulting rise in cAMP turns on the appropriate response in the cell by either changing the molecular activities in the cytosol, using protein kinase A (PKA), a cAMP-dependent protein kinase that phosphorylates target proteins or by turning on a new pattern of gene transcription as shown in Figure 1.6. This new pattern of gene transcription in cells occurs through the activity of cAMP response element-binding (CREB) proteins.

The transducin family of G proteins mediate signal transduction in the visual system by regulating specific forms of phosphodiesterase (PDE) which catalyse the metabolism of cyclic nucleotides (Neer, 1995; Wickman & Clapham, 1995). $G\alpha_t$ activates PDE via direct binding to the enzyme (Wickman & Clapham, 1995; Siegel et al., 1999). The ability of neurotransmitter receptors to stimulate the phosphoinositide second-messenger pathway is mediated by the activation of phosphatidylinositol-specific phospholipase C (PI-PLC), which catalyses the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to form the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) (Wickman & Clapham, 1995).

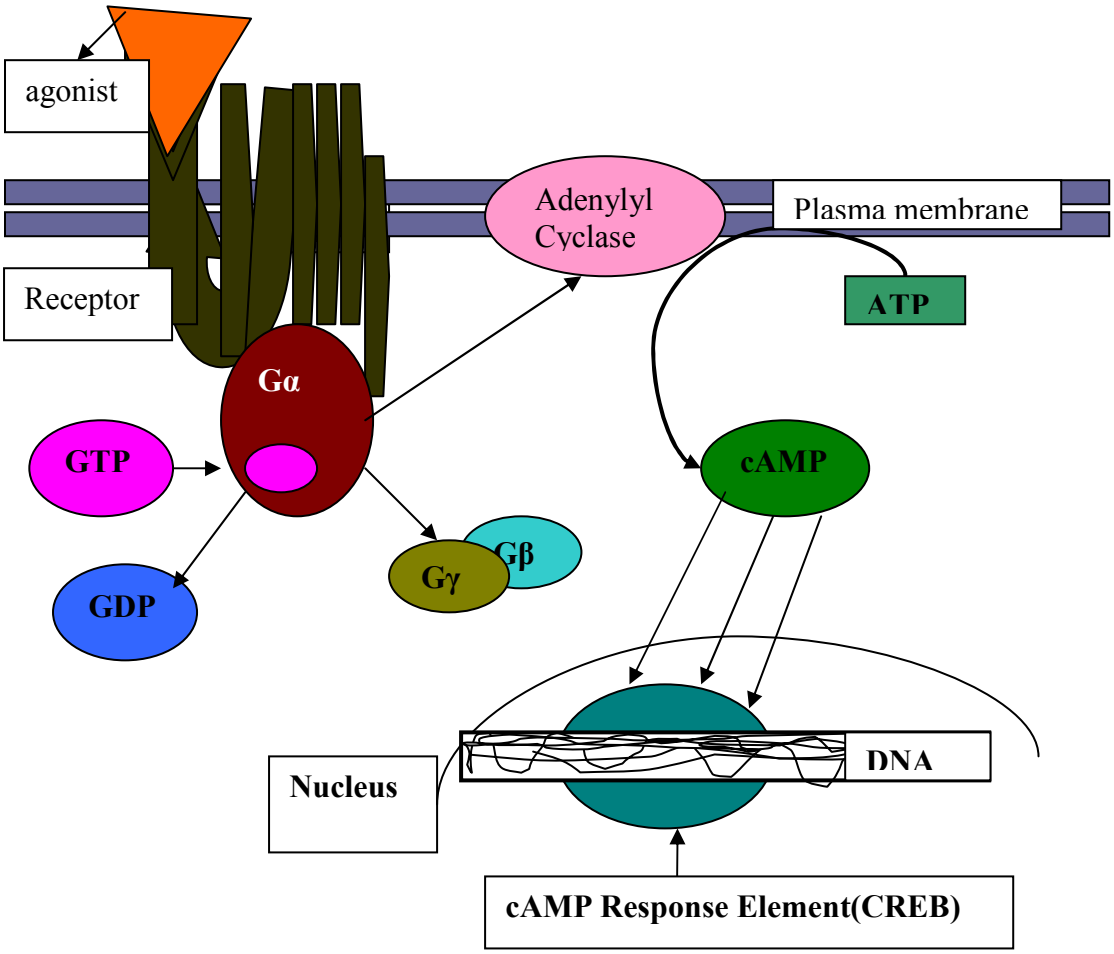


Fig.1.6. Representation of receptor activation resulting in the splitting of G protein complex into Gα and Gβγ leading to the phosphorylation of ATP to cAMP which pass into the nuclei as cAMP response element-binding proteins (CREB) and function as transcription factors of some genes. Adapted from Cabrera-Vera et al.(2003).

1.6. The nervous system

The mammalian nervous system is made up of the central nervous system (CNS) composed of the brain and the spinal cord, and the peripheral nervous system (PNS), which consists of the sensory-somatic nervous system and the autonomic nervous system. The brain receives sensory input from the spinal cord as well as from its own nerves which include olfactory and optic nerves, and devotes most of its volume and computational power, to processing its various sensory inputs and initiating appropriate and coordinated motor outputs (Siegel et al., 2006). The basic structural unit of the nervous system is the nerve cell or the neuron. Both the spinal cord and the brain consist of white matter bundles called axons which are each coated with a

sheath of myelin and grey matter which are masses of the nerve cell bodies, dendrites and synapses (Berridge, 2006).

A typical neuron is composed of three parts: a single cell body, many dendrites and a single axon. The dendrites receive signals from other neurons, the cell body integrates the signal and the axon conducts action potentials, and transmits signals to other neurons through the axon terminals. At the dendrite, the action potential can be elicited if the membrane potential is depolarized to a threshold (Berridge, 2006). The action potential then travels along the axon to the nerve terminal, where depolarization may open calcium channels for the entry of Ca^{2+} ions, which then induce the release of neurotransmitters stored in the vesicles (Kandel et al., 2000).

In a neural network, the nerve terminal of a neuron may form a synapse with either the dendrite or nerve terminal of another neuron. The synaptic cleft between two neurons is about 200 - 500 Å wide (Siegel et al., 2006). Chemical transmission is the major means by which nerves communicate with one another in the nervous system. The pre- and post-synaptic events are highly regulated and subject to use-dependent changes that are the basis for neuroplasticity and learning in the central nervous system (Siegel et al., 1999).

According to Siegel et al. (1999) and Berridge, (2006), chemical transmission requires

- synthesis of the neurotransmitter in the presynaptic nerve terminal.
- storage of the neurotransmitter in secretory vesicles
- a regulated release of neurotransmitter in the synaptic space between the pre- and post-synaptic neurons.
- the presence of specific receptors for the neurotransmitter on the post-synaptic membrane, such that application of the neurotransmitter to the synapse mimics the effects of nerve stimulation.
- a means of termination of the action of the released neurotransmitter.

Different types of substances act as neurotransmitters. Classical neurotransmitters like acetylcholine (ACh) and norepinephrine (NE) are low molecular weight substances that have no other function but to serve as neurotransmitters (Purves et al., 2001). The

predominant excitatory neurotransmitter in the brain, glutamate, and the inhibitory neurotransmitter in the spinal cord, glycine, are common and essential amino acids (Siegel et al., 1999). They function as neurotransmitters because the membranes of secretory vesicles in glutamatergic and glycinergic nerve terminals have specific transport systems that concentrate and store these amino acids so that they can be released by exocytosis in a highly regulated manner (Purves et al., 2001). Aminergic neurotransmitters, namely ACh and GABA, also enter synaptic vesicles through specific transport proteins. Neurotransmitters released from the vesicle may diffuse through the synaptic cleft to act on their receptors in the postsynaptic neuron as shown in Figure 1.7.

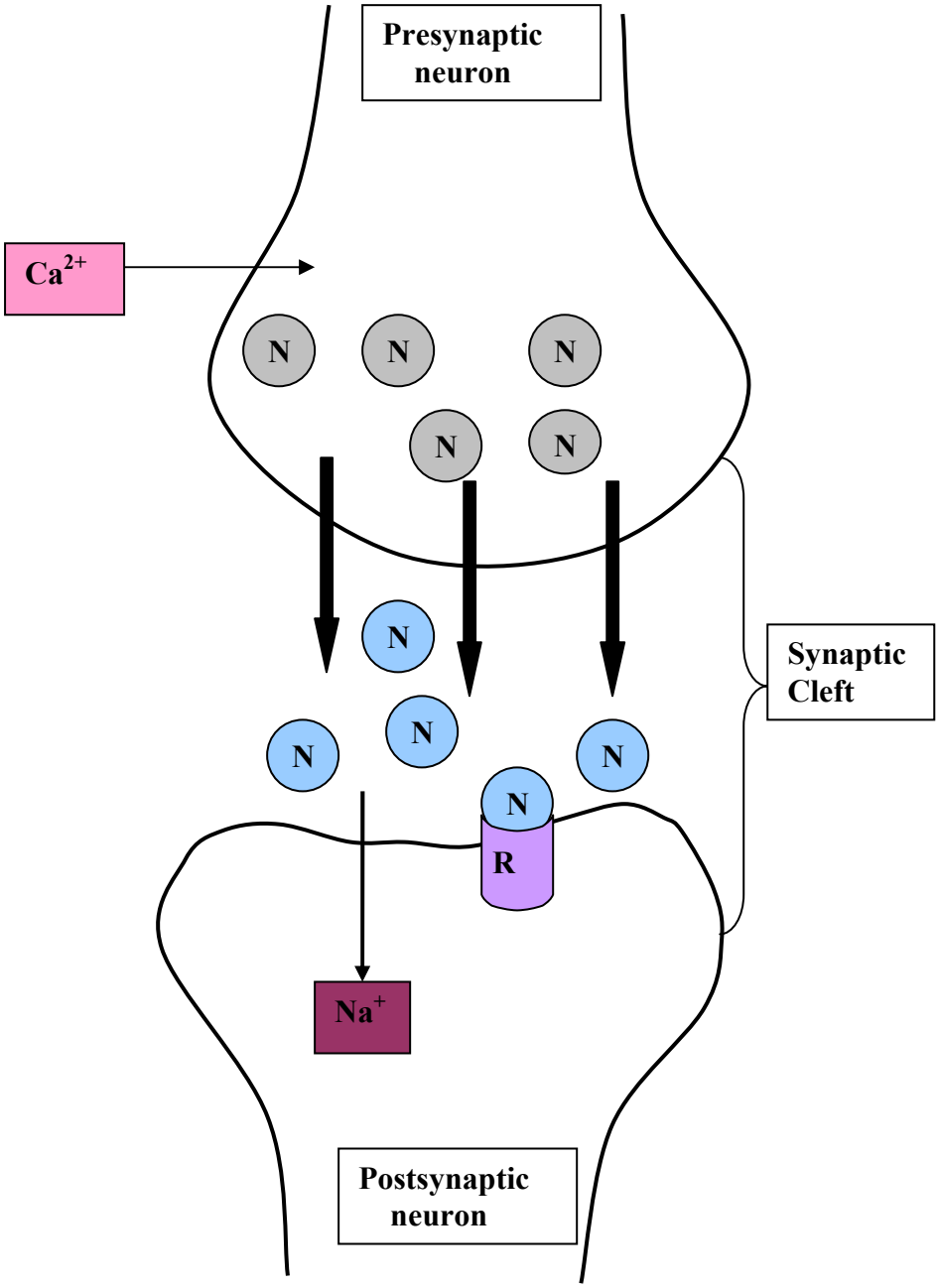


Fig.1.7. Mechanism of synaptic transmission. Neurotransmitters (N) are stored in vesicles at the presynaptic nerve terminal. The action potential at the presynaptic terminal causes the entry of Ca^{2+} ions through voltage-activated calcium channels, leading to the release of neurotransmitters (N) into the synaptic cleft which then bind to the receptors (R) with Na^{+} entry into the postsynaptic neurons through sodium channels. Adapted from Siegel et al.(2006).

The receptors for neurotransmitters can be classified into two categories: metabotropic receptors, also called GPCRs, and ionotropic receptors. GPCRs are involved in signal transduction. As previously described, binding of neurotransmitters

on GPCRs may trigger a series of signalling processes. The ionotropic receptors form ion channels which may be activated upon binding of specific neurotransmitters. Once activated, the influx of cations, such as Na^+, may cause the postsynaptic membrane to depolarize (Berridge et al., 2003). If the depolarization reaches the threshold, an action potential can be generated on the postsynaptic neuron. The ion channel formed by neurotransmitter receptors is called the synaptic channel. Each synaptic channel consists of five receptor subunits (Kandel et al., 2000).

1.7. Hypoxia

Hypoxia is a state of oxygen deficiency in a cell, tissue, a part or whole body which is sufficient to cause an impairment of function. Hypoxia is caused by the reduction in partial pressure of oxygen, inadequate oxygen transport, or the inability of the tissues to use oxygen. Hypoxia is also defined as a pathological condition in which the body as a whole (generalized hypoxia) or region of the body, tissues or cells (tissue or cellular hypoxia) is deprived of adequate oxygen supply. Symptoms of generalized hypoxia depend on its severity and speed of onset. They include headaches, fatigue, shortness of breath, nausea, unsteadiness, and sometimes even seizures and coma. This can be marked by reduced oxygen to parts of the body especially to brain and muscles (Guyton and Hall, 2005; Feriche et al., 2007).

Categories of hypoxia include the following:

- Hypoxic hypoxia, which is a reduction in the amount of oxygen passing into the blood. It is caused by a reduction in oxygen pressure in the lungs, a reduced gas exchange area, exposure to high altitude, or by lung disease, in which arterial oxygen pressure is reduced (Acker, 1996).
- Hypemic hypoxia also called anaemic hypoxia is a reduction in the oxygen carrying capacity of the blood. It is caused by a reduction in the amount of haemoglobin in the blood or a reduced number of red blood cells. A reduction in the oxygen transport capacity of the blood occurs through blood donation, haemorrhage, or anaemia, while a reduction in the oxygen carrying capacity of the blood occurs through drugs, chemicals, or carbon monoxide.

- Stagnant hypoxia also called ischaemic hypoxia is an oxygen deficiency due to poor circulation of the blood or poor blood flow (Dietz et al., 2003). Examples of this condition are high gravity forces, prolonged sitting in one position or hanging in a harness, cold temperatures, and positive pressure breathing.
- Histotoxic or cytotoxic hypoxia is the inability of the tissues or cells to obtain or use oxygen (Acker and Acker, 2004). Examples are in carbon monoxide and cyanide poisoning and in controlled tissues or cellular hypoxic experiments.

Cerebral hypoxia is a condition in which there is a decreased oxygen supply to the brain even though there is adequate blood flow (Guyton and Hall, 2005). Drowning, strangling, choking, suffocation, cardiac arrest, head trauma, carbon monoxide poisoning and complications of general anaesthesia can create conditions that can lead to cerebral hypoxia (Guyton and Hall, 2005; Kolb, 2003). Symptoms of mild cerebral hypoxia include inattentiveness, poor judgement, memory loss and a decrease in motor coordination (Kolb, 2003; Guyton and Hall, 2005). Neural and brain tissues have been shown to be extremely sensitive to oxygen deprivation and can begin to die within five minutes after oxygen supply has been cut off (Zhang et al., 2006; Tomaselli et al., 2005). When hypoxia lasts for longer periods of time, it can cause coma, seizures, and even brain death. In brain death, there is no measurable activity in the brain, although cardiovascular functions are preserved (Dietz et al., 2003). Hypoxia reoxygenation has been shown to induce premature senescence in neonatal Sprague-Dawley (SD) rat cardiomyocytes (Zhang et al., 2007).

Hypoxia in the brain may lead to cell death by apoptosis and necrosis. Tomaselli et al. (2005) have shown that adenosine, the final metabolic product of in the stepwise dephosphorylation of ATP, is produced and released in the central nervous system in response to ischaemia and hypoxia. Adenosine acts as a powerful endogenous neuroprotectant during ischaemia-induced energy failure by decreasing neuronal metabolism, increasing cerebral blood flow (via vasodilatation) and by playing a variety of different roles as an intercellular messenger (Tomaselli et al., 2005).

Most animals experience some degree of hypoxia and hypothermia during the course of their life as a consequence of ambient exposure or through metabolic, respiratory and circulatory insufficiency (Boutilier, 2001). Like humans, the vast majority of mammals are unable to survive prolonged periods of hypothermia and oxygen deprivation owing to irreversible membrane damage and loss of cellular ion homeostasis in vital organs like the brain and heart. However, numerous hibernating endotherms, neonatal and diving mammals as well as many ectotherms can tolerate prolonged periods and the key to their survival under such conditions lies in an inherent ability to down regulate their cellular metabolic rate to new hypo metabolic steady states in a way that balances the ATP demand and ATP supply pathways (Boutilier, 2001; Bunn and Poyton, 1996).

Most mammals possess little natural tolerance to severe hypoxia or hypothermia, and their excitable cells and tissues are normally debilitated by any prolonged exposure to either condition. The primary causes of hypoxia/anoxia-or hypothermia-induced death in mammals are brain dysfunction and cardiac arrhythmias due to a loss of ionic integrity of the cell membranes (Boutilier, 2001). Ion leakage across cell membranes occurs as a result of both intracellular and extracellular ions drifting towards their thermodynamic equilibrium (Rolfe and Brand, 1996; Priebe et al., 1996). Maintenance of a homeostatic intracellular environment therefore requires the redistribution of these ions through the use of ATP-dependent pumping systems such as the Na^+/K^+-ATPase, which can consume 20–80% of the cell's resting metabolic rate depending on the extent of its electrical activity (Edwards et al., 1989; Rolfe and Brown, 1997).

Cell death occurs when ATP production fails to meet the energetic maintenance demands of ionic and osmotic equilibrium. The rise in free cytosolic intracellular Ca^{2+} concentration results in the activation of Ca^{2+}-dependent phospholipases and proteases that further hasten the rate of membrane depolarisation, leading to uncontrolled cellular swelling and, ultimately, to cell necrosis (Hochachka, 1986; Boutilier, 2001).

In severe O_2 decrease, most mammalian excitable cells cannot continue to meet the energy demands of active ion-transporting systems, thus leading to rapid exhaustion of substrate which results in membrane failure and cell death (Willis, 1979). However, in certain lower vertebrates, neonates and diving mammals, hypoxia-induced membrane destabilisation of the kind seen in adult mammals is either slow to

develop or does not occur at all as a result of adaptive decreases in membrane permeability (i.e. ion 'channel arrest') that dramatically reduce the energetic costs of ion-balancing ATPases (Musacchina, 1984; Hochachka, 1986).

Mahura (2003), has shown that neuronal responses to hypoxia-ischaemia can be acute or chronic. In the early stages, neuronal responses to ischaemia-hypoxia are dependent on the modulation of ion channels. Acute responses rely mainly on O_2-regulated ion channels which mediate adaptive changes in neuron excitability. Energy failure, an early consequence of hypoxia-ischaemia, causes disruption of ionic homeostasis and accumulation of extracellular neurotransmitters. NMDA and AMPA/kainate receptors and Ca^{2+} channels contribute to excitotoxic neuronal degeneration. Excitotoxicity leads to increased Ca^{2+} influx, which can activate cytotoxic intracellular pathways (Mahura, 2003).

Reactive oxygen species generated during hypoxia-ischaemia contribute to the injury. Oxygen free-radicals serve as important signalling molecules that trigger inflammation and apoptosis (Durukan and Tatlisumak, 2007). Excitatory amino acid-receptor antagonists and Ca^{2+} channels blockers can provide neuroprotection in experimental models of hypoxia-ischaemia but disrupt normal brain function (Mahura, 2003; Durukan and Tatlisumak, 2007). The blockade of voltage-gated Na^{+} channels reduces the excitability of neurons, Na^{+} influx and the accumulation of intracellular Na^{+}, which improve the ionic homeostasis and cellular energy levels and prevent ischaemia-hypoxia induced neuronal injury and neuronal damage mediated by Ca^{2+} overload (Mahura, 2003). These findings from Mahura (2003) have shown that any neuroprotective agent against hypoxia and ischaemia should be able to reduce the generation of oxygen free radicals and limit Ca^{2+} influx into neuronal cerebral cells, which will invariably reduce inflammation and cell death. Thus any neuroprotective agent should also be able improve the ionic homeostasis and cellular energy levels and prevent ischaemia-hypoxia induced neuronal injury and neuronal damage mediated by Ca^{2+} overload (Mahura, 2003).

Prabhakaran et al. (2004) have shown that heterogeneous oxygen tension and access to metabolites in solid tumours may produce variability in response to adjuvant therapy. To better understand these micro environmental features, Prabhakaran et al.

(2004), examined survival and proliferation of neuroblastoma (NB) cells, in an *in vitro* model of hypoxia, and metabolite deprivation. They showed that human NB cells (SH-SY5Y) were subjected to a self-generated diffusion gradient of nutrient and oxygen deprivation in a modified *in vitro* sandwich model. In this model, the extent of both hypoxia and metabolite deprivation were individually altered, and the effects of each were studied.

Prabhakaran, et al. (2004) confirmed cellular proliferation using proliferating cell nuclear antigen (PCNA) immunocytochemistry and morphological assessment and hypoxia was assessed by vascular endothelial growth factor (VEGF) and pimonidazole immunocytochemistry. Prabhakaran et al. (2004) examined apoptotic cell death using terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis, assaying for plasma membrane transfer of phosphotidylserine and the presence of the anti-apoptotic protein Bcl-2 using immunocytochemistry. As predicted, cellular survival diminished with increasing duration and severity of hypoxia and metabolite deprivation. Oxygen deprivation was determined to be the more important contributory factor to early survival and proliferation. Proliferating cell nuclear antigen (PCNA) immunocytochemistry confirmed decreasing fractions of proliferating cells as a function of decrease in oxygen and metabolites. VEGF and Bcl-2 immuno-reactivity increased with prolonged exposure and increased extent of oxygen/metabolite deprivation. TUNEL analysis and phosphotidylserine transfer demonstrated cellular death of hypoxic and metabolite-deprived NB cells in a manner consistent with a mitochondrial apoptotic pathway (Prabhakaran et al., 2004). This *in vitro* model of hypoxia with NB cells demonstrates that increasing the severity of hypoxia and metabolite deprivation results in diminished proliferation and greater apoptotic cell death (Prabhakaran et al., 2004).

Gu and Shi (1994), also investigated the effect of hypoxia on the growth and differentiation of NG108-15 cells, hybrid cells derived by the fusion of mouse neuroblastoma clone N18TG6 with rat glioma clone C6BU1. These cells proliferate in the presence of foetal calf serum, but are differentiated by the addition of DbcAMP to the culture medium. The effect of hypoxia (2% O_2) on the hybrid cells, showed that hypoxia depressed proliferation of non-differentiated cells and induced death of differentiated cells and also inhibited differentiation in either non-differentiated or

differentiating immature cells, in the direction to look like mature neuro-like cells that could no longer be differentiated any more while some cells became large with flat short processes and had no Nissl substances (Gu and Shi, 1994). The observations of Prabhakaran et al.(2004) and Gu & Shi (1994) have shown that cellular hypoxia and metabolite deprivation lead to increased cellular death, decreased cellular growth, proliferation and differentiation, though both studies used established cell lines but differed in that Gu and Shi (1994) used hybrid cells. The effect of hypoxia in the two studies above was clearly shown, though the studies used established cell lines in contrast to primary cell lines where the effect of hypoxia would be more pronounced. This is because primary cultures may not survive for very long periods of time and as such may show more pronounced effects when compared to the established cell lines (Adler, 2006a).

1.7.1. Neuronal damage in hypoxia

Hypoxic stress has been shown to induce apoptosis of hippocampal CA1 neurons while selectively sparing CA2–3 neurons, while proliferation and differentiation of local stem cells may potentially replace lost neurons (Zhou et al., 2004). The mitogen activated protein (MAP) kinase mediated regulation of proliferation and differentiation was examined by Zhou et al.(2004) using rat organotypic hippocampal cultures exposed to hypoxia for up to 6 hours followed by reoxygenation. The results showed that the c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/ SAPKs) and extracellular signal–regulated protein kinases (ERKs) were maximally activated by 4 hours, returning approximately to basal levels by 6 hours (Zhou et al., 2004). Apoptosis of CA1 neurons was maximal by 6 hours hypoxia, although JNK activation had returned to basal levels. Zhou et al. (2004) demonstrated that a neuroprotective protein, JNK-interacting protein 1 (JIP1), an inhibitor of JNK-mediated apoptosis, was reduced by 6 hours of hypoxia and markedly decreased by 24 hours reoxygenation in CA1 neurons. This was the case for MAP-kinase activating death domain (DENN/MADD), which modulates JNK-mediated cell death (Zhou et al., 2004; Zhou and Miller, 2006).

The second peak of ERK1 activation occurred at 24 hours reoxygenation and declined to control levels by 48 hours. Zhou and Miller (2006) confirmed proliferation with anti-proliferation cell nuclear antigen (PCNA) immunohistochemistry and

Bromodeoxyuridine (BrdU) incorporation (Zhou et al., 2004). With an inhibitor of ERK activation, BrdU labelling was strikingly reduced, implicating ERKs in the proliferation response. This showed that, hypoxia concurrently triggered both JNK and ERK signalling, and with reoxygenation, ERK1 activation and stem cell proliferation followed by neuronal progenitor cell differentiation and targeted migration to the site of pyramidal neuronal loss (Zhou et al., 2004).

Pre-existing hyperglycaemia has been shown to exacerbate CNS injury after transient global and focal cerebral ischaemia (Kelleher et al., 1993; Vincent et al., 2005a). Increased anaerobic metabolism, with resultant lactic acidosis, has been shown to cause hyperglycaemic neuronal injury (Vincent et al., 2005a). The role of astrocytes in ischaemic-induced neuronal injury has been shown to be protective, and the ability of astrocytes to maintain energy status and ion homeostasis under hyperglycaemic conditions could ultimately reduce neuronal injury and hence increase neuronal survival rate (Kelleher et al., 1993; Vincent et al., 2005b).

Kelleher et al. (1993) studied the effects of increased glucose concentrations on glucose utilization, lactate production, extracellular pH and ATP concentrations in hypoxia-treated astrocyte cultures using primary astrocytes from neonatal rat cerebral cortices. After some days *in vitro*, cultures were incubated with 0-60mmol/L glucose and subjected to hypoxic conditions at 95% N_2/5% CO_2 for 24 hours. In addition, under high glucose (30mmol/L) conditions, astrocytes were exposed to up to 72 hours of hypoxia. Determination of lactate dehydrogenase efflux, adenosine triphosphate concentrations, and extracellular lactate concentrations was used to define the status of the astrocytes (Vincent et al., 2007).

The results of Kelleher et al. (1993) showed that when physiological concentrations of glucose (≤7.5 mmol/L) were used, significant cell damage occurred within 24 hours of hypoxia, as determined by increased efflux of lactate dehydrogenase and loss of cell protein. When higher glucose concentrations (15-60 mmol/L) were used, efflux of lactate dehydrogenase was similar to that observed in normoxic cultures, despite an increased utilization of glucose. Lactate concentrations in the media at low or normal glucose concentrations exceeded normoxic levels, but higher glucose concentrations (15-30 mmol/L), failed to increase lactate levels further (Kelleher et al., 1993; Vincent et al., 2005b). Values of ATP for hypoxic astrocytes treated with high glucose

concentrations were significantly higher than those of astrocytes with zero or low glucose levels (Vincent et al., 2007).

In cultures exposed to hypoxia and high glucose levels (30 mmol/L), no cellular injury was observed before 48 hours of hypoxia (Kelleher et al., 1993). Lactate dehydrogenase concentrations in the media increased during the first 24 hours of hypoxia and reached steady state, while the pH of the media was decreased after 24 hours and 48 hours. Injury occurred when the pH of the medium was less than 5.5. Vincent et al. (2005a) concluded that hypoxic injury to mature astrocytes was reduced by the presence of glucose (15-60 mmol/L) in the medium during 24-30 hours of hypoxia (Kelleher et al., 1993; Vincent et al., 2005a; Vincent et al., 2007).

To investigate whether high glucose concentrations protected cells by increasing extracellular osmolarity, Kelleher et al. (1993) added 0, 4.5, 7.5, 15 or 30 mmol/L mannitol in the medium containing 7.5 mmol/L glucose under normoxic and hypoxic conditions. The results showed that the increased osmolarity caused by mannitol had no protective effect on hypoxic cultures, nor did it affect the LDH release from normoxic cells (Kelleher et al., 1993). Vincent et al. (2005a) have shown that the application of 20mM added glucose but not 20mM non-metabolizable mannitol, significantly decreases aconitase activity in dorsal root neurons within 1 hour and continues to decrease activity over a 5 hour period. The above findings show that cellular protection afforded by high glucose concentrations is not due to increased osmolarity of the culture media in hypoxic and normoxic situations.

Brain ischaemia-induced neuronal death has been linked in part to excess Ca^{2+} influx through ionotropic glutamate receptors and voltage-gated Ca^{2+} channels (Choi, 1988; Rothman and Olney, 1986; Wei et al., 2003). As a result, considerable effort has been directed toward reducing neuronal circuit excitation as a strategy for neuroprotective intervention (Wei et al., 2003). Strategies of preventing neuronal death include direct blockade of *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors, while additional strategies include enhancing synaptic inhibition, reducing glutamate release, and reducing neuronal firing by blocking Na^+ channels or opening K^+ channels (Goldin et al.,1995; Taylor and Meldrum, 1995). The strategy of opening K^+ channels is supported by the observation that drugs that enhance opening of ATP-sensitive K^+ channels (K_{ATP}

channels) can reduce ischaemia-induced necrotic cell death in the heart (Grover,1997) and brain (Fujita et al.,1997). Also, the novel Ca^{2+}-activated maxi-K^+ channel opener, BMS-204352, was shown to be neuroprotective in rodent focal stroke models, (Gribkoff, et al., 2001), although clinical trial results have not been encouraging (Wahlgren and Ahmed, 2004; Goldberg, 2002; Goldberg, 2007).

It has been shown that excessive K^+ efflux and intracellular K^+ depletion are the key steps in the apoptotic cascade of many cells, including neurons (Dallaporta et al., 1998; Yu et al., 1997). One possible factor limiting the benefit of enhancing K^+ channel activity in the ischaemic brain might be enhancement of an apoptotic component of focal ischaemic neuronal death (Linnik et al., 1993; Choi, 1996; Wei et al 2003), a component that might be especially prominent when infarction is delayed after mild transient insults (Endres et al., 1998). Yu et al. (1999) observed that several apoptotic insults induced enhancements of the outward delayed rectifier currents in cultured cortical neurons which occurred before cells are committed to die, which is consistent with a potential role as an early mechanism in apoptosis.

A close relationship between enhanced K^+ channel activity and apoptosis was also supported by studies in other types of neurons and peripheral cells (Yu et al., 1999). The potassium channel blockers, tetraethyl ammonium (TEA) and clofilium, or elevated extracellular K^+, attenuated neuronal apoptosis in neurons and the neuroprotection persisted even when the concurrent Ca^{2+} influx and intracellular Ca^{2+} increase were prevented (Yu et al., 1999). TEA was found to be neuroprotective against CA1 hippocampal injury after global ischaemia in rats (Huang et al., 2001). Wei et al. (2003) investigated a possible role of K^+ channels in ischaemia-induced apoptosis and potential therapeutic implications of the K^+ hypothesis for apoptosis in ischaemic brain injury. They explored the protective effects of K^+ channel blockers against neuronal death induced by hypoxia *in vitro* and by mild transient focal ischaemia in the rat. Their result showed that 5 mmol/L of the K^+ channel blocker tetraethylammonium (TEA) selectively blocked K^+ channels without affecting NMDA receptor-mediated current or voltage-gated Ca^{2+} currents. These findings suggest that preventing excessive K^+ efflux through K^+ channels may constitute a therapeutic target for the treatment of stroke and other neurodegenerative disorders.

Sher and Hu (1992) investigated the comparative effects of rapid versus graded correction of chronic hypoxia *in vitro*. Cerebral cortical cell cultures obtained from foetal mice were exposed to 5% O_2 for 24 hours and returned immediately to room oxygen level for 24 hours. In a second group the cultures were exposed to 5% O_2 for 24 hours followed by 10% O_2 for another 24 hours and then returned to room oxygen level. At the end of the experiment the result showed that the partial pressure of oxygen in the bathing medium of the first group cultures was significantly higher than that of second group and non-hypoxic controls (Sher and Hu, 1992). Throughout the recovery period, the second group cultures showed improved neuronal survival, decreased lactate dehydrogenase efflux into the bathing medium and relative preservation of neuronal morphology (Sher and Hu, 1992). Glutamate binding was not differentially affected and glutamine synthetase activity, a predominantly glial marker, was only modestly increased after graded reoxygenation (Sher and Hu, 1992). These results demonstrated that gradual reoxygenation, after prolonged hypoxia *in vitro*, improves neuronal survival compared to rapid reoxygenation, and delays the manifestations of metabolic dysfunction even though the length of hypoxic exposure is increased. These results are in agreement with the concept that a period of relative hyperoxia may contribute to hypoxia-induced neuronal injury (Sher and Hu, 1992; Wei et al., 2003). This is because the initial exposure of cells to a hyperoxic environment will predispose the cells to severe hypoxic injury when they are eventually introduced to a hypoxic environment. The cells become highly susceptible to the injurious effects of hypoxia as a result of brief exposure to hyperoxia. Hu et al. (2003) have shown that the expression of two anti-apoptotic proteins (Bcl-2 and Bcl-X(L)) was decreased, and caspase 3 activity was increased significantly at 24 hour after hypoxia, and hypoxia plus hyperoxia. These results suggest that both hypoxia and hyperoxia alone can induce cell death, while cells initially exposed to hyperoxia before subsequent hypoxic exposure may be more predisposed to hypoxic injuries.

Bickler et al. (2003) showed that neonatal rats survived and avoided brain injury during periods of anoxia 25 times longer than adults and as such they hypothesized that oxygen activates and hypoxia suppresses NMDA receptor (NMDAR) responses in neonatal rat neurons. This explained the innate hypoxia tolerance of these cells. In CA1 neurons isolated from neonatal rat hippocampus with mean postnatal age of 5.8 days, hypoxia reduced NMDA receptor-channel open-time percentage, and NMDA-

induced a significant increase in Ca^{2+} and NMDA Delta Ca^{2+} by 38 and 68%, respectively. While in postnatal day 20 (P20) neurons, the reductions were not significant and in postnatal day 3 to 10 (P3-10) CA1 neurons within intact hippocampal slices, hypoxia reduced NMDA Delta Ca^{2+} by 52% and decreased NMDA-induced death by 45%. Phalloidin, a microtubule stabilizer, prevented hypoxia-induced inhibition of NMDA Delta Ca^{2+} in P3-10 neurons. Compared with responses in 21% O_2, hypoxia reduced currents from neonatal NMDA subtype NR1/NR2D receptors by 25%, increased currents from NR1/NR2C by 18%, and had no effect on NR1/NR2A or NR1/NR2B NMDA receptor subtypes (Bickler et al., 2003). Modulation of NMDARs by hypoxia may play an important role in the hypoxia tolerance of the mammalian neonate, and oxygen sensing by NMDARs could play a significant role in postnatal brain development (Bickler et al., 2003).

Normally, neurotransmitter levels are highly regulated by the nervous system. In certain medical conditions, such as Parkinson's disease and Alzheimer's disease, injured nerve cells become unable to control the normal release of neurotransmitters and they tend to release excess glutamate into the extracellular environment (Friedman, 2006; Hedrick et al., 2005). The excess glutamate results in over excitation of the NMDA receptor allowing excess calcium to enter the affected neurons (Tremblay et al., 2000). These neurons may then swell and rupture, releasing more glutamate into the surrounding area, which in turn overexcites NMDA receptors on adjacent neurons. This cascade of neuronal injury, referred to as excitotoxicity, follows acute conditions such as stroke and traumatic brain injury (Hasbani et al., 2001; Bickler et al., 2004). Damaged nerve cells also appear to release excess glutamate in certain chronic conditions such as neuropathic pain and dementia (Rivera-Cervantes et al., 2004; Schiller et al., 2000).

Molecular mechanisms underlying foetal growth restriction due to placental insufficiency and *in utero* hypoxia are not well understood, but in the study by Huang et al. (2004), time-dependent changes in foetal tissue gene expression in a rat model of *in utero* hypoxia compared with normoxic controls were investigated as an initial approach to understand molecular events underlying foetal development in response to hypoxia. The result showed that litter size was reduced and insulin-like growth factor binding protein 1 (IGFBP-1) was up-regulated in maternal serum and in foetal liver and

heart (Huang et al., 2004). It has been shown that in hypoxia, there is the reduction in foetal growth, brought about by the decrease in the availability of growth factors resulting in reduced litter size. The significance of this finding is that, in hypoxia, there is growth hormone deficiency which leads to elevated IGFBP-1 levels (Marchini et al., 2005). This is because increasing stress and energy demands as in the case of hypoxia are accompanied by increasing foetal levels of glucose-mobilizing hormones in combination with depressed levels of insulin and IGF-I, and increase in IGFBP-1 is aimed at diminishing insulin-like activity of IGF-I, thereby reducing peripheral glucose utilization and hence the reduction in growth (Marchini et al., 2005).

Tissue-specific, distinct regulatory patterns of gene expression were observed under acute and chronic hypoxic conditions and the induction of glycolytic enzymes was an early event in response to hypoxia during organ development (Huang et al., 2004). Tissue-specific induction of calcium homeostasis-related genes such as L-type voltage-gated calcium channel (rattus norvegicus calcium channel alpha-1D subunit; ROB3) and glutamate receptor, ionotropic, kainate 4 (Grik4), and suppression of growth-related genes like brain-derived neurotrophic factor (BDNF) and nerve growth factor-induced factor A (NGFI-A) were observed, suggesting mechanisms underlying hypoxia-related foetal growth restriction. Also the induction of inflammation-related genes, such as immunoglobulin superfamily number 6 (Igsf6) and prostaglandin-endoperoxide synthase 2 (Ptgs2), in placentas exposed to long-term hypoxia, suggest that a mechanism for placental dysfunction and impaired pregnancy outcome accompanying *in utero* hypoxia (Huang et al., .2004).

1.7.2. Morphological and functional changes in hypoxia

Transient cerebral ischaemia has been shown to lead to delayed neuronal dysfunction which may occur in hours and days after ischaemic insult (Lipton, 1999; Zhong et al., 2003). Pathological conditions could result, to a variable extent, in cell damage or even death depending on the severity of the insult (Lushnikova et al., 2004; Mander et al., 2005). Strong ischaemia has been shown to lead to quick pronounced neuronal death while mild insults might cause less remarkable cell damage (Jourdain et al., 2002).

Culture models of neurons from developing rat forebrain in moderate hypothermia, concomitant with a hypoxic insult, have been shown to exert protective effects on nervous cells and prevent apoptosis (Bossenmeyer-Pourie et al., 2000). Ischaemia has been suggested to be a common risk factor in Alzheimer's Disease and Huntington's disease, epilepsy and other neurodegenerative diseases (Afsar et al., 2003; Egashira et al., 2002). Egashira et al. (2002) have shown that hypoxia enhances β-amyloid-induced apoptosis in rat cultured hippocampal neurons. While Bossenmeyer et al. (1998) have shown that hypoxia for 6 hours reduced neuronal viability by approximately 40% within 96 hours after reoxygenation, in line with the appearance of morphologic features of apoptosis, such as nuclear condensation and fragmentation. Such alterations are preceded by typical and reproducible changes in the rates of total protein synthesis, reflected by a biphasic increase in leucine incorporation taking place 1 hour after the onset of hypoxia and also 48 hours after reoxygenation. Thereafter, protein synthesis progressively declined, concomitantly with the appearance of cell damage and a reduction of the number of viable neurons (Bossenmeyer-Pourie et al., 2000; Haddad and Jiang, 1993).

Apoptotic cell death generally occurs in a tightly regulated manner that proceeds mainly through highly conserved genetic mechanisms (Steller, 1995). Earlier studies have shown that delayed cell death resulting from a 6-hour period of hypoxia in cultured neurons is accompanied by an increased expression of pro-apoptotic gene products, such as Bax and members of the family of cysteine proteases referred to as caspases, including CPP32/caspase-3 (Bossenmeyer et el., 1998). Preconditioning has been shown to protect neuronal cells against hypoxia-induced apoptosis by stimulating the induction of specific cell survival-associated genes (Bossenmeyer-Pourie et al., 2000). Adaptive mechanisms may include over expression of the inducible isoform of heat shock protein (HSP) 70 and Bcl-2 proteins, whereas others have reported the production of trophic factors, such as basic fibroblast growth factor (bFGF) in rat cortical neurons (Sakaki et al., 1995). Both HSP 70 and Bcl-2 have been directly implicated in the regulation of the apoptotic process and are largely recognized as anti-apoptotic effectors (Tamatani et al., 1998; Yang et al., 1997).

It has been shown that when neuronal cells are exposed to hypoxia for 6 hours they respond by early and transient induction of HSP 70, but the protein level progressively

decreases, while preconditioned neurons are able to maintain high amounts of HSP 70 until the experimental end point (Bossenmeyer-Pourie et al., 2000). Preconditioning also induces expression of Bcl-2, the prototypic repressor of apoptotic death, which functions by counteracting the death promotor activity of some of its conserved homologs, such as Bax (Oltvai et al., 1993) and may additionally contribute to inhibition of apoptosis by preventing the posttranslational activation of caspase members (Srinivasan et al., 1996; Allen et al.1998). Shimizu et al. (1996) reported that, although Bcl-2 and caspase inhibitors are efficient in attenuating hypoxia-induced neuronal death, they might also be involved in the reduction of at least some types of necrotic damage. Bossenmeyer-Pourie et al. (2000) have shown that hypoxia induces neuronal death mainly through an apoptotic process, but the rate of necrosis appears to increase significantly during the course of the experiment. Although this may reflect necrosis secondary to apoptosis, necrotic damage may partly account for hypoxia-induced cell loss. In conditioned neurons, the final number of necrotic cells after hypoxia remains in the range of control cells, suggesting that if it does not reduce basal necrosis observed during the normal life-span of cultured neurons, preconditioning might suppress the necrotic component of hypoxic injury (Bossenmeyer-Pourie et al., 2000).

Hypothermia has been shown to promote the early post ischaemic recovery to normal glucose utilization in rats subjected to transient global cerebral ischaemia (Bossenmeyer-Pourie et al., 2000). Temperature reduction contributes to reducing the release of neurotransmitters, especially glutamate, which mediates excitotoxicity (Globus et al., 1995). Neuronal preconditioning before a hypoxic insult appears to trigger adaptive cell responses via active mechanisms that include the production of anti-apoptotic molecules. Probably through its depressant actions on cell activity, hypothermia impairs active processes that are necessary for the cells to undergo apoptosis, and possibly secondary necrosis, in response to hypoxia-reoxygenation (Bossenmeyer-Pourie et al., 2000).

1.7.3. Hypoxic disorders

Hypoxia has been shown to regulate growth, proliferative capacity and collagen type-1 production in cardiac fibroblasts and, therefore, may play a part in the post-infarct remodelling of the cardiac collagen matrix (Agocha et al., 1997). Inflammation and myocardial injury occurs as a result of apoptosis of cardiomyocytes (Gottlieb et al., 2003). Apoptosis has been shown to represent the major independent form of myocyte death during, and after, infarction (Kajstura et al., 1996). Hypoxia has been shown to induce apoptosis in many cell types (Tanaka et al., 2001). Apoptosis has been induced in rat myocardium within 45 minutes of ischaemia and this was shown to advance substantially during 3 hours of reperfusion (Black et al., 2005).

Apoptosis found in hypoxic cardiomyocytes has been found to be concomitant with raised protein 53 (p53) activity, a transcription factor that regulates cell cycle and functions as a tumour suppressor, indicating a critical role for p53 activated pathways (Long et al., 1997). However, it has been demonstrated that hypoxia-induced apoptosis seen in acute myocardial infarction also occurs in the absence of p53 (Bialik et al., 1997). Diagnosis of acute myocardial infarction is facilitated by raised levels of the enzymes lactate dehydrogenase (LDH) and creatinine kinase (CK), levels of which increase in response to hypoxia/reoxygenation, along with a corresponding increase in levels of nitric oxide (NO) (Wei and Zhu, 1997). The vasodilatory effect of nitric oxide contributes to the inflammatory response, thereby leading to reperfusion injury (Wallace and Ma, 2001).

The production of the nitric oxide is catalysed by the enzyme nitric oxide synthase (NOS) and the most common form, when considering cardiac ischaemia, is endothelial NOS (eNOS). This has been shown to be produced by blood vessel endothelia and is responsible for the synthesis of endothelium-derived relaxing factor (EDRF). The two other enzyme forms are cytokine-inducible NOS (iNOS) and the neuronal form of nitric oxide synthase (nNOS) (Polster and Fiskum 2004). It is known that nitric oxide is released from cardiomyocytes in response to hypoxia/ reoxygenation (Wei and Zhu, 1997; Polster and Fiskum, 2004) and a decreased production of endothelial nitric oxide by both acute and chronic hypoxia has been observed in pulmonary arteries (Cokkinos et al., 2005; Ikonomidis et al., 2005). Pacher et al. (2007) have shown that mammalian cells are able to generate a large

amount of reactive oxygen species (ROS) in response to oxidative stress. These ROS include oxygen ions, free radicals and peroxides. The reactive oxygen ions can react rapidly with nitric oxide to form peroxynitrite ($ONOO^-$), which is an oxidizing agent that can damage cells and their DNA (Pacher et al., 2007).

Cells exposed to a hypoxic environment are radio- and chemo-resistant, and these resistant cells can remain viable after cancer treatment, leading to the possibility of recurrence and metastasis on cessation of therapy (Airley et al., 2000). In addition, the adaptations necessary to maintain viability of these cells in hypoxic conditions may cultivate a more malignant phenotype. There is some evidence that hypoxia favours the selection of aberrant p53 tumour suppresser gene (Graeber et al., 1996; Weinmann et al., 2004a), and that exposure to hypoxia increases tumour growth rate and experimental metastatic potential (Stackpole et al., 1994; Weinmann et al., 2004b; Cooper et al., 1998). Cells cultured in anoxic conditions are approximately three times more resistant to the cytotoxic effects of radiation than fully aerated cells, with radio-sensitivity rapidly increasing with increasing oxygen concentration (Airley et al., 2000; Serkova et al., 2002).

Radio-resistance occurs due to the deficiency of molecular oxygen, which acts as a radiosensitiser due to its action as an electron acceptor. As a consequence, the free radical formation that results in cell damage occurs to a much lesser extent under hypoxic conditions, leading to survival of these dangerous cells (Weinmann et al., 2004a; Seta et al., 2002; Semenza, 2007). Hypoxia also confers changes in radio-sensitivity due to the proliferative, physiologic and metabolic changes associated with cells exposed to this adverse microenvironment (Rockwell, 1992; Knisely and Rockwell, 2002; Semenza, 2007).

1.8. Neuroprotection in hypoxia

Hypoxia has been known to generate ROS, the by-products of cellular oxidative metabolism (Adams, 1979; Halliwell and Gutteridge, 1989). It has been shown that of all the cells in the body, the neurons are highly vulnerable to ROS damage and ROS may play an important role in the processes leading to neuronal cell damage due to

hypoxia (Ratan et al., 1994; Jayalakshmi et al., 2005). The high vulnerability of brain cells to oxidative injury is due to their high metabolic rate (which hypoxia disrupts), elevated levels of polyunsaturated lipids (a target of lipid peroxidation) and low levels of natural antioxidants and anti-oxidative enzymes such as superoxide dismutase (Reiter, 1995; Warner et al., 2004).

Jayalakshmi et al. (2005) have shown that neurons from rat hippocampus exposed to transient hypoxia, showed increased oxidative stress and decreased antioxidant enzyme defence system. The administration of N-acetyl cysteine (NAC) resulted in significant neuroprotection against oxidative stress induced by the hypoxia. Research is being directed towards identifying and characterizing proteins that maintain neuron survival in the normal brain, as well as those that, when altered by hypoxia and mutation, result in neuronal oxidative stress and even death (Shaul et al., 1993; Cavanaugh et al., 2005). Although many of these proteins are found specifically in neurons, some are located in other brain cells such as astrocytes, oligodendrocytes and cells within the cerebrovascular system (Halliwell and Gutteridge 1989; Silver and Erecinska, 1998). These proteins are potential targets for drugs that could be used to prevent or treat neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, multiple sclerosis (MS) and stroke, which may lead to the design of novel drugs that are effective against neurological and neurotoxic disorders (Cavanaugh et al., 2005).

Mohanakumar et al. (2000) investigated the hydroxyl radical ($^{\cdot}OH$) scavenging action and neuroprotective effects of salicylate (SA) in mice treated with neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Their study showed that MPTP treatment resulted in *in vivo* generation of $^{\cdot}OH$ and nigral neuronal insult as evidenced by dopamine depletion in nucleus caudatus putamen (NCP). They showed that MPTP caused a significant reduction in glutathione, an antioxidant that protects cells against toxins such as free radicals, in the substantia nigra (SN) and NCP. The administration of salicylate alone in mice did not affect total monoamine oxidase (MAO) activities in the mitochondrial fraction or the crude enzyme preparation from SN or NCP (Mohanakumar et al., 2000).

Pre-treatment of the animals with SA, resulted in the dose-dependent production of dihydroxybenzoic acid (DHBA) in NCP, while the administration of SA before and

after MPTP treatment, blocked the neurotoxin-induced behavioural dysfunction, (glutathione and dopamine depletion) which indicates the neuroprotective action of SA (Mohanakumar et al., 2000). The results suggest that SA acts as a free radical scavenger in the brain and indicates its strength as a valuable neuroprotectant (Mohanakumar et al., 2000). Though the results from the study by Mohanakumar et al. (2000) showed that SA could act as a free radical scavenger and a potential neuroprotective agent, the study was conducted using a whole animal experiment which may not produce the same effect when these are studied in an isolated cellular or tissue system as in a culture model. This is due to the fact that the body could have put together different defensive systems to produce the observed effects, compared to that seen in a culture system composed of one cell type.

Studies are continuously being conducted in collaboration with researchers within the clinical, neuroscience and neuroprotection research groups in universities and hospitals to identify and develop clinically useful neuroprotective agents. The work of life sciences companies such as the Neurobiological Technologies, Inc. (NTI®) are focused on the development of neuroprotective agents, some of which have been shown to protect nerve cells from injury in laboratory experiments and are pending their clinical evaluation and final approval. It has been shown that in multiple nervous system disorders, including neuropathic pain and AIDS-related dementia, over activation of the NMDA receptors on the membranes of neurons leads to neuronal injury, resulting in progressive neurological impairment (Cavanaugh et al., 2005). Thus, drugs that modulate or inhibit the activity of NMDA receptors restore progressive neuronal impairment and thereby improve neurologic symptoms such as chronic pain, motor difficulties, memory loss and other cognitive deficits. Such agents would, therefore, be a valuable treatment for a number of serious neurological disorders (Cavanaugh et al., 2005).

1.9. Opioid receptor agonists

The term opioid refers to any natural or synthetic drugs that have morphine-like activity. They are classified as natural, semi-synthetic and synthetic opioids. Examples of natural opioids are morphine, codeine noscopine; semi-synthetic are heroin, oxymorphone and hydromorphone; while the synthetic opioids are methadone, morphinians and benzamorphans (Piestrzeniewicz et al., 2006).

Opioid receptors are found in the central nervous system and are classified as mu (μ), kappa (κ), delta (δ) and sigma (σ) opioid receptors. Opioid receptors are not uniformly distributed in the CNS and are found at areas concerned with pain receptors, with the highest concentration in the cerebral cortex, followed by the amygdala, septum, thalamus, hypothalamus, midbrain and spinal cord (Raynor et al., 1996; Chaturvedi et al., 2000). The mu receptor has been shown to be high in areas of pain perception and in the medulla, especially in the area for respiration (Reisine and Bell 1993; Reisine and Brownstein 1994; Massotte and Kieffer 1998; Hasbi et al., 2000).

The opioid receptors (mu, delta, and kappa) belong to the large family of GPCRs and have diverse and important physiological roles (Piestrzeniewicz et al., 2006; Rhim and Miller 1994). Laugwitz et al.(1993) have shown that activated delta opioid receptors are coupled to G_{i1} while activated mu opioid receptors are coupled to G_{i3} in neuroblastoma cells (SH-SY5Y). Mu opioid receptors have been shown to be activated by mu receptor agonists and are coupled through the $G\alpha_{i1}$ and $G\alpha_{oA}$ in human embryonic kidney (HEK 239) cells (Saidak et al., 2006). Tso and Wong (2000), have shown that both mu and kappa opioid receptors are coupled via both G_i and G_z in HEK 239 cells. The opioid receptors are important targets for thousands of pharmacological agents (Hasbi et al., 2000; Wang et al., 2007). The stimulation of these receptors triggers analgesic effects and affects the function of the nervous system, gastrointestinal tract and other body systems (Piestrzeniewicz et al., 2006). The discovery of opioid peptides (including delta-selective enkephalins, kappa-selective dynorphins, and mu-selective endomorphins), which are endogenous ligands of opioid receptors, initiated their structure-activity relationship studies (Fichna et al., 2006).

Piestrzeniewicz et al. (2006), have shown that in the last 30 years, hundreds of analogs of opioid peptides have been synthesized in an effort to make the compounds more active, selective, and resistant to biodegradation than the endogenous ligands. Different unnatural amino acids, as well as cyclisation procedures, leading to conformationally restricted analogs, were employed. All these modifications resulted in obtaining very selective agonists and antagonists with high affinity at mu-, delta-, and kappa-opioid receptors, which are extremely useful tools in further studies on the pharmacology of opioid receptors in a mammalian organism (Piestrzeniewicz et al., 2006; Xiong et al., 2007). GPCRs typically require activation or stimulation by agonists for their signalling activity to be initiated but Wang et al. (2007), have shown that some of the GPCRs display basal or spontaneous signalling activity in the absence of an agonist. This basal or spontaneous signalling activity is also called constitutive activity (Wang et al., 2007; Piiper and Zeuzem, 2004).

As mentioned, opioids exert their biological activity through the activation by GPCRs, and their effects can be blocked by receptor antagonists. Opioid antagonists with different inverse agonist properties have different effects in precipitating withdrawal in acute morphine dependent mice, and constitutive opioid receptor activation is critically involved in acute opioid withdrawal (Freye and Levy, 2005; Wang et al., 2007; Xiong et al., 2007). It has been shown that the pharmacological properties and activities of the three opioid receptor classes are distinct and can be clearly differentiated (Raynor et al., 1996). Opioid receptors have high affinity for both agonists and antagonists as shown from Table 1.3.

DAMGO and its antagonists do not bind to delta or kappa receptors, and morphine and its derivatives are much less potent at the delta or kappa receptors. All the three opioid receptors are sensitive to the antagonist naloxone (Raynor et al., 1996; Raynor et al., 1994; Wang et al., 2007).

Table 1.3. Some agonists and antagonists of opioid receptor subtypes

Opioid receptor subtype	Agonists	Antagonists
Delta	Deltorphin- Penicillamine-2,Penicillamine-5-enkephalin (DPDPE) [D-serine 2, O-Leu5]-enkephalin-Thr (DSLET) TAN-67 D-Ala^2]-Deltorphin II	Naltrindole ICI 174,864 Dalargin SDM25N hydrochloride Naltriben mesylate ICI 154,129 Benzylnaltrindole-hydrochloride
Kappa	U50,488 [3H]U69,593 ([3H]U69) ICI 204,448 (ICI) ICI-199441hydrochloride U-54494A hydrochloride BRL 52537-hydrochloride	nor-binaltorphimine (nor-BNI) 7-benzylidenenaltrexone (BNTX) [3H]diprenorphine ([3H]DIP) GNTI dihydrochloride
Mu	(D-Ala^2-MePhe^4, Gly-ol^5) enkephalin (DAMGO) Morphine Loperamide-hydrochloride Endomorphin-1 Endomorphin-2	D-Phe-Cys-Tyr-D-Trp-Orn-Pen-Thr-NH2 (CTOP) Naloxonazine Cyprodime hydrochloride H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2. (CTAP)

1.9.1. Uses of opioids

Opioids have long been used to treat acute pain, such as post-operative pain (Raynor et al., 1994). They are commonly prescribed, and used, because of their effective analgesic properties. Studies have shown that properly managed medical use of opioid analgesic compounds is safe and rarely causes addiction. Taken exactly as prescribed, opioids can be used to manage pain effectively. They have also been found to be invaluable in palliative care to alleviate the severe, chronic and disabling pain of terminal conditions such as cancer and AIDS (Doyle et al., 2004). Contrary to popular belief, high doses are not required to control the pain of advanced or end-stage disease. In recent years there has been an increased use of opioids in the management of non-malignant chronic pain. This practice has grown from over 30 years experience in palliative care of long-term use of strong opioids, which has shown that dependence is rare when the drug is being used for pain relief (Doyle et al., 2004)

In addition to analgesia, clinical uses of opioids include: codeine and hydrocodone for cough, natural opioids for diarrhoea; oxymorphone for anxiety due to shortness of breath; and methadone and buprenorphine for heroin detoxification and maintenance programs during heroin replacement therapy (Eap et al., 1999; Eap et al., 2002). Despite the fact that opioids have been extensively reported to have psychological benefits, they are never officially prescribed to treat psychological illnesses, even in circumstances where researchers have reported opioids to be especially effective (e.g. in the treatment of senile dementia, geriatric depression, and psychological distress due to chemotherapy or terminal diagnosis) (Berridge, 2006).

Doyle et al. (2004) have shown that opioids are used to treat pain of moderate or greater severity, irrespective of the underlying pathophysiological mechanism. Morphine has been used to treat breathlessness of which several mechanisms have been suggested for its action. Codeine and loperamide are the most widely used opioids for diarrhoea. Loperamide has the advantage of acting only on the gut, since very little is absorbed and topical morphine in an aqueous gel can be an effective agent for treatment of painful wounds. Their use is based on the discovery of activated opioid receptors in damaged tissue (Doyle et al., 2004). Opioid medications can affect regions of the brain, resulting in the initial euphoria that many opioids produce. They can also produce drowsiness, cause constipation, and, depending upon the amount taken, depress breathing. When taken as a large single dose, opioids could cause severe respiratory depression or death (Wang et al., 2007).

1.9.2. Opioid receptor activation

Milligan (2004) has shown that the opioid receptors form homomeric as well as heteromeric receptor complexes. Opioid receptors are capable of forming a heterodimer with each other and certain non-opioid receptors, for example, mu with $alpha_{2a}$-adrenoceptors (Devi, 2001). This heterodimerisation between opioid receptors has been shown to result in changes in the pharmacology of the receptors as well as changes in receptor coupling to second messengers and trafficking (Corbett, et al., 2006). It has been shown that both mu and delta receptors internalise on exposure to agonists, whereas kappa receptors do not and when such dimers such as delta/kappa

dimers are formed, the trafficking properties of the kappa receptor predominates, while the heterodimer does not internalise on exposure to agonists of either receptors (Corbett et al., 2006; Koch & Hollt, 2007). Opioid receptor subtypes have been proposed largely on the basis of radioligand binding studies and as such there is little or no evidence for the presence of the different genes encoding these subtypes but in some cases, receptor heterodimerisation of opioid receptors has been proposed as a possible explanation (Corbett et al., 2006).

All of the opioid receptors are GPCRs and couple to their cellular effectors primarily through G_i/G_o proteins, and thus the majority of opioid responses are pertussis toxin-sensitive (Milligan & Kostenis, 2006). Corbett et al. (2006), have shown that the different behaviours mediated by each of the receptor subtype in the intact animal such as euphoria for mu and dysphoria for kappa, result not from each type of receptor evoking different cellular responses but from the different anatomical distributions of each receptor (Corbett et al., 2006). Although the predominant action of opioids in the nervous system is inhibitory, in several brain regions such as periaqueductal grey (PAG) (important for supraspinal analgesia) or ventral tegmental area (VTA) (for euphoria/reward), opioids are excitatory (Corbett et al., 2006). It has been shown that opioid-induced excitations are due, not to a direct excitatory action of opioids, but to disinhibition (Corbett et al., 2006). The apparent excitation of a neuron by opioids is as a result of inhibition of the release of inhibitory neurotransmitters such as gamma amino butyric acid (GABA) from the interneurons into the cell (Corbett et al., 2006).

Opioids have been shown to act via receptors interacting with heterotrimeric pertussis toxin (PTX) sensitive G proteins. The mu-selective agonist, DAMGO, and the delta-selective agonist, [D-Pen^2,D-Pen^5]-enkephalin (DPDPE) stimulated the incorporation of the photo-reactive GTP analogue into proteins co-migrating with the alpha subunits of G_{i1}, G_{i2}, G_{i3}, G_{o1}, and G_{o2} in the membranes of neuroblastoma SH-SY5Y cells while in the membranes of PTX-treated cells, both agonists were ineffective, because mu and delta opioid receptors appear to discriminate between PTX-sensitive G proteins which lead to activation of distinct G protein subtypes (Laugwitz et al., 1993). Subtype-specific immuno-precipitation of G protein alpha subunits photo-labelled in the absence or presence of agonists revealed profound differences between

mu and delta opioid receptors in coupling to PTX-sensitive G proteins (Milligan, 2004).

Opioid inhibition of neuronal excitability resulting in the down-regulation of pain occurs largely by activation of potassium channels in the plasma membrane (Samways & Henderson, 2006). Opioid receptors are now known to activate a variety of potassium channels, including G-protein-activated inwardly rectifying (GIRK), calcium-activated inwardly rectifying, dendrotoxin-sensitive and M-type channels (Williams et al., 2001). Opioid receptors have been shown to inhibit high threshold voltage-activated calcium channels, like other members of the G_i/G_o-coupled receptor family such as cannabinoid (CB_1) receptors (Corbett et al., 2006). In some cell types, such as neuronal cells, opioid receptor activation can also cause an elevation of the free calcium concentration inside cells by releasing calcium from intracellular stores or by enhancing calcium entry by a dihydropyridine-sensitive mechanism (Samways & Henderson, 2006). It has been shown that the activation of opioid receptors, stimulates a variety of intracellular signalling mechanisms including activation of inwardly rectifying potassium channels, and inhibition of both voltage-operated N-type Ca^{2+} channels and adenylyl cyclase activity (Samways & Henderson, 2006). It is now apparent that like many other G_i/G_o-coupled receptors, opioid receptor activation can significantly elevate intracellular free Ca^{2+}, although the mechanism underlying this phenomenon is not well understood (Samways & Henderson, 2006). In some cases opioid receptor activation alone appears to elevate intracellular Ca^{2+}, but in many cases it requires concomitant activation of G_q-coupled receptors, which themselves stimulate Ca^{2+} release from intracellular stores via the inositol phosphate pathway (Samways & Henderson, 2006).

Opioid receptors, like other G_i/G_o-coupled receptors, inhibit adenylyl cyclase resulting in a fall in intracellular cAMP (Corbett et al., 2006). Williams et al., (2001), have shown that in primary afferent neurons, opioid receptors activate and regulate multiple second messenger pathways associated with effector coupling, receptor trafficking, nuclear signalling and modulates the activation of hyperpolarization-activated cation channels. In opioid withdrawal, cAMP levels are elevated and enhanced protein kinase A (PKA) activity increases neurotransmitter release (Corbett et al., 2006). Opioid receptors, like many other GPCRs, cycle to and from the plasma

membrane from intracellular compartments (Corbett et al., 2006). This cycling is caused by agonist activation of the receptors which results in the cycling of the receptors to the plasma membrane in response to various stimuli (Williams et al., 2001). The generally accepted mechanism underlying mu and delta receptor desensitization, is that agonist-activated receptors on the plasma membrane are phosphorylated by G-protein-coupled receptor kinases (GRKs), which facilitates arrestin binding and prevents the receptor from coupling to G-proteins (Bailey & Connor, 2005). Arrestin-bound receptors are rapidly concentrated in clathrin-coated pits and undergo dynamin-dependent internalisation into early endosomes (Corbett et al., 2006). Delta receptors are trafficked into lysosomes and are down-regulated, whereas mu receptors are trafficked into endosomes, where they are dephosphorylated and recycled back to the plasma membrane in a re-sensitised state (Corbett et al., 2006). Thus, for mu receptor, internalisation can be considered to be involved in re-sensitisation, but not in desensitisation and there is evidence that different C-terminus splice variants of the mu receptor re-sensitise at different rates while the kappa receptors do not appear to internalise in response to agonist activation (Corbett et al., 2006).

Chakrabarti et al. (1995), have suggested that different G-proteins can be activated with different potencies by mu receptor agonists in Chinese-hamster ovary cell membranes, which agrees with studies on delta- and kappa-opioid receptors, suggesting that commonly used agonists of these receptors can activate multiple G-protein subtypes with similar potency (Burford et at., 2000). Carter and Medzihradsky (1993), have shown that mu-selective agonist, DAMGO inhibited cAMP formation in membranes of human neuroblastoma cells (SH-SY5Y), differentiated with retinoic acid. Antibodies to G_i alpha 1, 2 or G_i alpha 3 reduced the mu-opioid signal insignificantly and inhibition of adenylyl cyclase by the delta-opioid agonist (DPDPE) was very sensitive to the G_i alpha 1, 2 antibodies (Carter and Medzihradsky, 1993).

1.10. Cannabinoids

Cannabinoids are a group of terpenophenolic compounds present in *Cannabis sativa L.* and are made up of three types, namely natural or herbal cannabinoids, synthetic cannabinoids and endogenous cannabinoids (ElSohly and Slade, 2005). Cannabinoids were first characterised in the 1930s and 1940s as active ingredients of the cannabis plant (marijuana plant) from where the name was derived (Gardner, 2006). Some 68 natural plant cannabinoids have been described to date with Δ^9- tetrahydrocannabinol being shown as the main psychoactive component (ElSohly and Slade, 2005), and since the 1970s, many synthetic compounds have been developed by various laboratories to exert effects like those of the plant cannabinoids. These are the synthetic agonists and to block such effects are the antagonists (Gardner, 2006).

Three main cannabinoids found in the cannabis plant are cannabidiol (CBD) (Baker et al., 1980; Lewis et a., 2005), Δ^9-tetrahydrocannabinol (Δ^9-THC) (Gaoni and Mechoulam, 1964; Stefanidou et al., 1998), and cannabinol (CBN) (Gambaro et al., 2002). The amount and relative abundances of these three main cannabinoids has been used to characterize cannabis and this also varies according to geographical origin (Hillig and Mahlberg, 2004). It was believed that Δ^9-THC produced its effect by perturbing neuronal cell membranes due to its lipid-soluble, hydrophobic nature (ElSohly and Slade, 2005). However, the structural and steric selectivity of the actions of Δ^9-THC and its synthetic analogues suggested the involvement of receptors (Begg et al., 2005) This was later demonstrated by studies that documented the existence of saturable, stereo-selective, high affinity membrane-binding sites for cannabinoids in the mammalian brain (Begg et al., 2005).

In the 1990s, several chemicals in the body were identified as acting similarly and are called endocannabinoids or endogenous cannabinoids. Cannabinoid receptors are concentrated mainly in the cerebellum and the basal ganglia, the areas of the brain responsible for motor control which may help explain why marijuana eases muscle spasticity in disorders like multiple sclerosis, as well as in the hippocampus, which is responsible for storage of short-term memory, and the amygdala, which is part of the limbic system involved in emotional control, memory of fear, and memory of pain (Gardner, 2006; Begg et al., 2005). These receptors are now known as CB_1 receptors and are predominant in the central nervous system (Matsuda et al., 1990; Soderstrom

and Johnson, 2000).

A second cannabinoid receptor was initially detected in spleen cells, white blood cells, and other tissues associated with the immune system. This second receptor is called the CB_2 receptor and is mainly present in the peripheral system (Munro et al., 1993). William Devane and colleagues were the first to identify the original endogenous cannabinoid called arachidonyl ethanolamine (AEA), which they named "anandamide" (Devane et al., 1992). The AEA, is a brain-derived lipid that binds to cannabinoid receptors and mimics the biological effects of Δ^9-THC (Begg et al., 2005). Some years later, the second endocannabinoid was isolated from the intestinal tract and brain, called 2-arachidonoylglycerol (2-AG) (Sugiura et al., 1995).

Subsequently, several other related lipids with endocannabinoid properties have been identified however, these have been less extensively characterized (Begg et al., 2005). The biological effects of endogenous, plant-derived and synthetic cannabinoids are mediated through specific G protein coupled cannabinoid (CB) receptors. The CB_1 receptor is highly conserved in mice, rats and humans while the CB_2 receptors are more divergent (Begg et al., 2005). Both CB_1 and CB_2 receptors are coupled through $G_{i/o}$ proteins to inhibit adenylyl cyclase and regulate calcium (↓) and potassium (↑) channels (Begg et al., 2005; Mackie, 2006). In tissues naturally expressing CB receptors and in transfected cell lines, CB_1 and CB_2 receptors have been shown to have a high level of ligand-independent activation (Begg et al., 2005). It has been shown that in the population of wild-type CB_1 receptors, only about 30% exists in the activated form while 70% are inactive (Kearn et al., 1999; Begg et al., 2005; Carter and Weydt, 2002). Some of the CB_1 receptors exist in inactivated form within the cytosol and are in GDP-bound state while some exist in a tonically activated state, and are coupled to active G-proteins within the plasma membrane in their GTP-bound state (Vásquez and Lewis, 1999; Nie and Lewis, 2001). It has been shown that in their activated state, the receptors have a higher affinity for the cannabinoid agonists (Vásquez and Lewis, 1999; Nie and Lewis, 2001). Therefore, the cannabinoid receptors exist predominantly in two states: the activated and inactivated forms, of which they show differences in their affinities to their agonists and ligands (Kearn et al., 1999; Nie and Lewis, 2001).

The expression of the CB_2 receptor is more restricted and is limited primarily to immune and haematopoietic cells (Munro et al., 1993). The human CB_2 receptors show 68% amino acid homology with the CB_1 receptor in the transmembrane domains and a 44% overall homology (Munro et al., 1993; Begg et al., 2005). However, despite the low level of homology between the two receptors, their pharmacology is similar, with most plant-derived, endogenous and classical synthetic cannabinoids having similar affinities for the two receptors (Showalter et al., 2005; Begg et al., 2005), although synthetic agonists with greater than 100-fold affinity for CB_1 or CB_2 receptors have been developed (Hillard et al., 1997; Malan et al., 2001). The CB_1 receptors are highly conserved in mice, rats and humans while the CB_2 receptors are much more divergent. The amino acid homology of CB_2 between mouse and rat is 93% while that between rat and human is only 81% (Munro et al., 1993; Griffin et al., 2000). Both CB_1 and CB_2 receptors are the primary targets of endogenous cannabinoids and they play important role in many processes, including metabolic regulation, craving, pain, anxiety, bone growth, and immune function (Mackie, 2006). It has been shown that various intracellular kinases, including the mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases type 1 and 2 (ERK 1&2), C-Jun N-terminal kinase (JNK), focal adhesion kinase and protein kinase B/Akt, are also activated by both CB_1 and CB_2 receptors (Derkinderen et al., 2001; Derkinderen et al., 2003; Bouaboula et al., 1999).

There is also evidence of agonist selectivity for CB_1 receptors coupled to different subtypes of G_i proteins or to G_i versus G_o proteins (Howlett, 2004). Cannabinoid-activated receptors distinct from CB_1 or CB_2 have been postulated to exist in the central nervous system (Begg et al., 2005). Cannabinoids are known to inhibit GABA-mediated inhibitory postsynaptic currents (IPSCs) in the hippocampus via a presynaptic action at CB_1 receptors located on GABAergic terminals (Wilson et al., 2001). CB_1 receptors have also been implicated in the inhibition of glutamatergic excitatory postsynaptic currents. The synthetic cannabinoid, Win 55,212-2, a mixed CB_1-CB_2 cannabinoid receptor agonist, was found to attenuate hyperalgesia in a rat model of neuropathic pain, and suppress opioid-induced emesis in ferrets (Bridges et al., 2001; Simoneau et al., 2001).

1.10.1. Therapeutic potential of cannabinoids

Cannabis and its derivatives have a great therapeutic potential and have been used for centuries for medicinal purposes. However, cannabinoid-derived drugs on the market today lack specificity and produce many side effects, thus limiting their therapeutic usefulness (Pertwee, 2008). These side effects include euphoric mood changes, acute psychotic episodes, initiation and exacerbation of schizophrenic psychosis in predisposed persons, impaired cognitive and psychomotor performance, tachycardia and hypotension (Pertwee, 2008). The production of complex behavioural effects by cannabinoids are mediated by cannabinoid receptors (CB_1 and CB_2), and by interactions with other neurochemical systems (Adams and Martin, 1996; Carter and Weydt, 2002).

It has been shown that the therapeutic and physiological effects of cannabinoids are dependent upon whether the administration is acute or chronic and on the route of administration (Halpin et al., 1998; Fride et al., 2004). The physiological effects of cannabis and its derivatives include: reduction in psychomotor coordination and performance, alterations in thermoregulation, endocrine and reproductive functions and gut motility (Martins et al., 2006; Fride et al., 2004 ; Jackson et al.,2004). The therapeutic uses of some cannabinoid agonists and antagonists are shown in Table 1.4

Table 1.4 Therapeutic uses of cannabinoid agonists and antagonists

Agonist	Antagonist
Dronabinol (Marinol), an analogue of Δ^9-THC as anti-emetic agents in cancer therapy	SR141716 (Rimonabant), a selective cannabinoid (CB_1) receptor antagonist used as an anti-obesity drug
Nabilone (Cesamet), a synthetic cannabinoid and an analogue of Marinol reduces pain, an appetite stimulant and increases general well being in AIDS	SR141716 (Acomplia) is also used for the treatment of tobacco addiction
Sativex, a cannabinoid extract oral spray containing both Δ^9-THC and CBD, use for neuropathic pain and spasticity	SR141716A attenuated Δ^9-THC- or anandamide-induced memory impairment and attenuated the anandamide-induced impairment of performance.
Cannabichromene (CBC), an anti-inflammatory agent.	
Cannabidiol(CBD),a major anti-convulsant, anti-spasmodic, anti-asthmatic and anti-glaucoma agent	
Other effects of CB agonist include antinociceptive effects in animal models of acute inflammatory and neuropathic pain.	

The active ingredient of cannabis, Δ^9-THC, and other cannabinoids and their derivatives are being used to treat a variety of disorders (Baker et al., 2003). Drugs which selectively activate CB_1 and CB_2 receptors which include dronabinol and nabilone (synthetic analogues of Δ^9-THC) are used for the treatment of nausea and vomiting caused during cancer chemotherapy treatments (Martins et al., 2006). Many cannabinoids produce inhibition of pain responses and there is laboratory evidence to support the analgesic effect of cannabinoids (Martins et al., 2006**;** Martinez-Orgado et al., 2003). Other therapeutic uses of cannabinoid receptor agonists may include the suppression of some symptoms associated with multiple sclerosis, with spinal injury and with certain other movement disorders such as muscle spasticity and spasm, and the management of glaucoma, bronchial asthma, pain and inflammatory disorders (Halpin, 1998; Adams and Martin, 1996; Martins et al., 2006;Garcia-Arencibia et al., 2007).

The CB_1 receptor antagonist, SR141716A (rimonabant; Acomplia®), may have therapeutic potential in reducing memory deficits associated with ageing or neurological diseases (Pertwee,1997; Halpin, 1998). There is evidence that

cannabinoids are effective in relieving spasticity, tremor and pain caused by multiple sclerosis or spinal injury (Croxford, 2003; Pertwee, 2005; Pertwee, 2008). Animal experiments have shown that cannabinoid receptor agonists suppress spinal reflexes, produce marked behavioural changes in motor function, for example hypokinesia and catalepsy, and have significant efficacy in standard tests of antinociception (Halpin et al.,1998; Martins et al., 2006). In a clinical trial in healthy volunteers, using 2%, 4%, and 8% Δ^9-THC by weight on pain induced by capsaicin injected 5 and 45 mins after drug exposure, pain and hyperalgesic effects were assessed. The results show that by 45 mins after cannabinoid exposure, there was a significant decrease in capsaicin-induced pain with the medium dose and a significant increase in capsaicin-induced pain with the high dose (Wallace et al., 2007).

The effects on motor function are mediated by large populations of cannabinoid CB_1 receptors that are present in the basal ganglia of the brain, but whether they produce their putative antispasticity effect by acting at these brain sites remain to be established (Halpin et al., 1998; Neitzel and Hepler, 2006). Experiments have shown that Δ^9-THC can delay the onset and reduce the intensity of the clinical signs of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. It has also been shown that the synthetic cannabinoid receptor agonist, Win55212-2, can decrease the severity of dystonia in mutant Syrian hamsters with primary generalized dystonia (Herzberg et al., 1997). These indicate that cannabis, Δ^9-THC or nabilone can reduce the intensity of some signs and symptoms of multiple sclerosis or spinal injury, particularly spasticity, pain, tremor and nocturia (Baker et al., 2003). There is substantial evidence to suggest that various cannabinoids possess analgesic properties, but most of this evidence is based on experiments in laboratory animals. Results of studies which have employed clinically relevant models of inflammatory or neuropathic pain are now appearing and generally support the concept of cannabinoid-induced analgesia (Herzberg et al., 1997; Martins et al., 2006; Gilbert et al., 2007; Jackson et al., 2004).

Cannabinoids can alleviate tremor and spasticity in animal models of multiple sclerosis and clinical trials of the use of these compounds for these and other symptoms are continuously in progress (Croxford, 2003; Felder et al., 2006; Makriyannis, 2007). Anecdotal evidence has shown that patients with disorders such

as multiple sclerosis smoke cannabis to relieve disease-related symptoms and pains (Croxford, 2003; Diaz-Laviada and Ruiz-Llorente, 2005). Evidence has also shown that cannabinoids may prove useful in Parkinson's disease by inhibiting the excitotoxic neurotransmitter, glutamate, and counteracting oxidative damage to dopaminergic neurons (Halpin et al., 1998; Russo and McPartland, 2003). The inhibitory effect of cannabinoids on reactive oxygen species, glutamate and tumour necrosis factor suggests that they may be potent neuroprotective agents (Croxford, 2003; Croxford and Miller, 2003). Dexanabinol (HU-211), a synthetic cannabinoid is currently being assessed in clinical trials for traumatic brain injury and stroke (Croxford, 2003).

Animal models of mechanical, thermal and noxious pain suggest that cannabinoids may be effective analgesics (Croxford, 2003; Dikic et al., 2003 ; Docagne et al., 2007). Cannabinoids have proved more effective than the placebo in clinical trials of post operative and cancer pain and pain associated with spinal cord injury. However, they may be less effective than existing therapies (Russo, 2001; Docagne et al., 2007). Dronabinol, a commercially available form of Δ^9-THC, has been used successfully for increasing appetite in patients with HIV wasting disease and cannabinoid receptor antagonists may reduce obesity (Russo and McPartland, 2003). Acute adverse effects following cannabis usage include sedation and anxiety. These effects are transient and may be less severe than those that occur with existing therapeutic agents (Russo, 2003). Russo (2001), have shown that the use of nonpsychoactive cannabinoids such as cannabidiol and dexanabinol may allow the dissociation of unwanted psychoactive effects from potential therapeutic benefits. Rog et al. (2007) and Nurmikko et al. (2007) have demonstrated the analgesic property and efficacy of Sativex, an oro-mucosal Δ^9-THC/CBD endocannabinoid modulator compound against neuropathic pain caused by multiple sclerosis in humans.

The existence of additional cannabinoid receptors may provide novel therapeutic targets that are independent of CB_1 receptors, and the development of compounds that are not associated with CB_1 receptor-mediated adverse effects (Russo, 2001). In a study to investigate the therapeutic benefits and adverse effects of prolonged use of medical marijuana in a cohort of chronically ill patients, the result demonstrated clinical effectiveness in these patients in treating glaucoma, chronic musculoskeletal

pain, spasm and nausea, and spasticity of multiple sclerosis (Russo, 2001; Russo, 2003). There has been substantial evidence from experiments with animals, healthy human subjects and patients with primary open-angle glaucoma that cannabinoids can lower intra-ocular pressure (Green, 1998; Halpin et al., 1998 ; Russo, 2003).

Cannabinoids have shown great promise for the treatment of early phase response of asthma (Halpin et al., 1998). This is because they can significantly dilate the bronchioles of both healthy and asthmatic subjects and seem to be no less effective than conventional drug treatment of asthma (Hollister, 1986; BMA, 1997; Halpin et al., 1998). It has been shown that both cannabis and individual cannabinoids are active when taken orally or when inhaled, either in smoke or in an aerosol produced by a nebulizer inhaler (Hollister, 1986; BMA, 1997). The mechanism behind the bronchodilator effect of cannabinoids remains to be established. However, only cannabinoids with psychotropic properties have been found to produce bronchodilation, indicating that the effect may be mediated through cannabinoid CB_1 receptors.

Cannabis and cannabinoids, like all other drugs, have unwanted effects, as reported by Pertwee (1997), in a clinical study with 34 cancer patients, and include dizziness, sedation and dry mouth, blurred vision, mental clouding, ataxia, disorientation, disconnected thought, slurred speech, muscle twitching and impaired memory. Cannabis on its own may sometimes induce transient confusion, panic attacks, depersonalization, paranoid delusions and hallucinations (Chaudry et al., 1991) and has been reported to produce a subtle impairment of postural control (Pertwee, 1997). Cannabis may aggravate existing psychoses and can elevate heart rate; hence it would be unwise to give psychotropic cannabinoids to patients with schizophrenia, coronary arteriosclerosis or congestive heart failure (Hollister, 1986; Pertwee, 1997).
The list of some known cannabinoid agonists and antagonists used in experimental studies is shown in Table 1.5.

Table 1.5. List of some known cannabinoid agonists and antagonists used in experimental studies.

Type	Agonist	Antagonist
CB_1	Win 55,212 mesylate	SR141716A
	CP 55940	AM 281
	HU-210	AM 251
	Δ^9-tetrahydrocannabinol (Δ^9-THC)	Tetrahydrocannabivarin (THCV)
	Δ^8- tetrahydrocannabinol (Δ^8-THC)	LY 320135
	Cannabinol (CB)	
	Leelamine hydrochloride	
	Arachidonoylethanolamide (Anandamide or AEA)	
	2-arachidonylglycerol (2-AG)	
	arachidonyl-2-chloroethylamide (ACEA)	
	methanandamide	
	Arachidonylcyclopropylamide (ACPA)	Cannabidiol (CBD)
CB_2	AM-1241	SR144528
	JWH-015	AM 630
	JWH-133	
	CB 65	JTE 907
	L-759,633	

1.10.2. Neuroprotection by cannabinoids

Cannabinoids have been shown to provide neuroprotection in acute and chronic neurodegeneration (Lastres-Becker et al., 2004; Jentsch et al., 1998). In a study to examine the effect of cannabinoids against the toxicity caused by 6-hydoxydopamine both *in vivo* and *in vitro*, it was found that the non-selective cannabinoid agonist HU-210 increased cell survival in cultures of mouse cerebellar granule cells exposed to the toxin. However, the effect was significantly less when cannabinoids were directly added to neuronal cultures than when these cultures were exposed to conditioned medium obtained from mixed glial cell cultures treated with HU-210, suggesting that the cannabinoid exerted its major protective role by regulating glial influence to neurons (Lastres-Becker et al., 2004; Pryce et al., 2003 ; Drysdale et al., 2006).

Cannabinoids may also be neuroprotectant in Parkinson's disease (PD), a motor neurodegenerative disorder characterised by progressive death of nigrostriatal dopaminergic neurons that mainly results in bradykinesia or slowness of movement, rigidity, and tremor as major motor abnormality (Sethi, 2002; Lastres-Becker et al., 2005). In an experiment to investigate if cannabinoids might provide neuroprotection in PD, Lastres-Becker et al.(2005), conducted two sets of experiments to demonstrate that cannabinoids are effective against the *in vivo* and *in vitro* toxicity of 6-hydroxydopamine, a toxin currently used to generate Parkinsonism in laboratory animals. In the first experiments, Lastres-Becker et al. (2005), examined the ability of Δ^9-THC and cannabidiol (CBD), to alter *in vivo* the progress of neurodegeneration in rats subjected to unilateral injections of 6-hydroxydopamine into the medial forebrain bundle. In the second experiments, Lastres-Becker et al. (2005), evaluated whether the termination of Δ^9-THC administration to 6-hydroxdopamine-lesioned rats after 2 weeks would result in re-initiation of the process of neuronal injury during two subsequent weeks. This experiment also examined whether the potential effects of Δ^9-THC against *in vivo* toxicity of 6-hydroxydopamine are mainly neuroprotective because they do not disappear after discontinuation of cannabinoid treatment. The results from the studies by Lastres-Becker et al. (2005), showed that the daily administration of Δ^9-THC for 2 weeks produced a significant increase in dopamine content and tyrosine hydroxylase activity in the lesioned striatum and these were accompanied by an increase in tyrosine hydroxylase-mRNA levels in the substantia nigra. This suggests a potential neuroprotective action of cannabinoids against the *in vivo* and *in vitro* toxicity caused by 6-hydroxydopamine, which might be relevant in Parkinson's disease.

Cannabinoids have been shown to protect against neurotoxicity in a number of different cellular, animal and human experimental models (Davies et al., 2002; Fride and Shohami, 2002; Mechoulam et al., 2002; Pryce et al., 2003; Zhuang et al., 2005). Zhuang, et al.(1999) had earlier demonstrated that cultured rat hippocampal neurons were protected from excitotoxic insults by pre-treatment with either Δ^9-THC or Win 55,212-2, and that these compounds were effective in preventing cell death even if administered prior to the neurotoxin exposure. Cannabinoids have been demonstrated to be protective *in vivo* with respect to neurodegeneration resulting from experimental

ischaemia (Zhuang et al., 2005; Molina-Holgado et al., 2005). Leker et al.(2003) have shown that *in vivo* administration of CB_1 agonist HU-210, was able to significantly reduce motor disability and infarct volume after focal irreversible cerebral ischaemia. The mechanisms involved in the neuroprotective properties of cannabinoid have not yet been fully characterised (Mechoulam et al.,2002; Baker et al., 2003; Zhuang et al., 2005).

Zhuang et al. (2001) have shown that cannabinoid receptor-mediated neuroprotection is sensitive to intracellular calcium levels. In their work, Zhuang et al. (2002), presented a detailed analysis of how cannabinoids act to reduce or block release of intracellular calcium $[Ca^{2+}]i$ under neurotoxic conditions. They demonstrated that such neuroprotection is based on cannabinoid CB_1 receptor-mediated decreases in cAMP-dependent protein kinase (PKA), an effect that alters the sensitivity of particular intracellular calcium channels. Several possible alternative signalling pathways were also investigated and systematically ruled out on the basis that they did not block the NMDA provoked increase in $[Ca^{2+}]i$ in the same manner as CB_1 receptor activation (Ryan et al., 2007). It has been shown that there is a similar time course for the protective effect on cultured neurons, the blockade of intracellular calcium release and the inhibition of PKA (Zhuang et al., 2005). Common factors underlying these changes are the alteration in sensitivity of type-II ryanodine receptor (RyR)-coupled intracellular calcium channels and the decrease in cAMP due to cannabinoid inhibition of adenylyl cyclase, as originally demonstrated by Howlett et al. (1990).

Most of these protectant effects appear to be mediated by activation of the cannabinoid CB_1 receptor subtype (Parmentier-Batteur et al., 2002), although additional mechanisms may be involved (Lastres-Becker et al., 2005). It has been shown that the same neuroprotective effect is also produced by cannabidiol (CBD), another plant-derived cannabinoid, with negligible affinity for cannabinoid CB_1 receptors (Pertwee, 1999; Ryan et al., 2006), suggesting that antioxidant properties of both compounds might be involved in these *in vivo* effects. However, an alternative explanation might be that, the neuroprotection exerted by both compounds is due to their anti-inflammatory potential (Lastres-Becker et al., 2005; Jentsch et al., 1998; Jackson et al., 2004).

Cannabinoids' anti-inflammatory properties are likely related to their ability to modulate glial influence on neurons (Walter and Stella, 2004; Lastres-Becker et al., 2005; Marchalant et al., 2007). These anti-inflammatory properties might be important in Parkinson's disease (PD) since nigral cell death is accompanied by astrocyte proliferation and reactive microgliosis at the sites of neurodegeneration. Microglial activation may play important role in the initiation and early progression of the neurodegenerative process especially in regions which are particularly rich in microglia and other glial cells (Gao et al., 2002; Lastres-Becker et al., 2005; Louw et al., 2000).

It is shown that activated microglia produce a wide array of cytotoxic factors, including tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), eicosanoids, nitric oxide and reactive oxygen species that impact on neurons to induce neurodegeneration and that some of them have been reported to be increased in the substantia nigra and the caudate-putamen of PD patients (Gao et al., 2002).

1.11.3. The role of cannabinoids in neuroinflammation

Inflammation is a complex biological response and active defence reaction of tissues against harmful stimuli and insults (Walter and Stella, 2004). Inflammation is designed to remove or inactivate noxious agents and to inhibit their detrimental effects (Walter and Stella, 2004). It has been shown that inflammation, even though it serves a protective function in controlling infections and promoting tissue repair, can also cause tissue damage and disease (Walter and Stella, 2004; Lu et al., 2005). There is growing evidence that a major physiological function of cannabinoid signalling is to modulate neuroinflammation. The anti-inflammatory properties of cannabinoids have been studied at the molecular, cellular and whole animal levels, by examining the evidence for anti-inflammatory effects of cannabinoids obtained using *in vivo* animal models of clinical neuroinflammatory conditions, especially rodent models of multiple sclerosis (Walter and Stella, 2004; Jackson et al., 2004; Marchalant et al., 2007).

CNS inflammations occur in myelin degenerative disorders such as multiple sclerosis (MS), Alzheimer's disease, HIV encephalopathy, ischaemia and traumatic brain

injury (Martino et al., 2002). Experimental autoimmune encephalopathy (EAE) has been shown to be a widely used animal model to study MS owing to histological similarities (Zamvil and Steinman, 1990). The EAE is initiated and maintained as a result of T lymphocytes becoming sensitized to myelin proteins and eliciting a cell-mediated immune response. The pathological changes involve demyelination and a progression of inflammation in the CNS (Walter and Stella, 2004). Cannabinoid administration has been shown to influence the course of the disease progression on several studies performed on various rodent models of MS (Matsuda et al., 1990; Matsuda, 1997; Munro et al., 1993). In animals administered with Δ^9-THC prior to inoculation with lymphocytes from CNS of animals with acute EAE, full clinical development of EAE was prevented, suggesting that that Δ^9-THC suppressed the immune system (Lyman et al., 1989). In animals given Δ^9-THC after inoculation with the lymphocytes, onset of symptoms was delayed and clinical index was lowered, while histological examination of spinal cords showed significantly less inflammation in Δ^9-THC treated animals (Lyman et al., 1989).

Wirguin et al. (1994), administered delta-8-tetrahydrocannabinol (Δ^8-THC), a minor form of THC, daily to rats with EAE beginning several days prior to symptoms onset. The Δ^8-THC treated animals had a delayed symptom onset, lowered incidence of EAE and a shorter mean duration of EAE but not a lower mean severity. In mice with EAE, a synthetic cannabinoid receptor agonist (Win 55212-2), THC, methanadamide, and selective CB_2 receptor agonist (JWH-133), but not cannabidiol, relieved the spasticity and tremor symptoms within 1 to 10 minutes (Baker et al., 2000). The effects of Win 55212-2 were reversed by treatment with the selective cannabinoid receptor antagonists SR141716A (CB_1) and SR144528 (CB_2) and these two compounds administered alone worsened symptoms (Baker et al., 2000). Since the CB_2 receptor agonist and a CB_2 receptor antagonist influenced these symptoms, these results point towards an anti-inflammatory effect mediated via CB_2 receptors, which are expressed mainly on immune cells (Walter and Stella, 2004).

The effects of cannabinoid compounds over several weeks were studied on a model of human multiple sclerosis (MS) in which the cannabinoid agonists Win 55212-2, arachidonyl-2-chloroethylamide (ACEA), and a CB_2 selective agonist JWH-015 were administered daily for 10 days following Theiler's murine encephalomyelitis virus

(TMEV) infection of the CNS. This induces an immune-mediated demyelinating disease in susceptible mouse strains, but prior to symptoms (Walter and Stella, 2004). These drugs improved motor function, decreased the number of activated microglia in the spinal cord, decreased major histocompactibility complex class II (MHC II) expression, decreased the number of CD4 T cells in spinal cord, and promoted spinal cord remyelination (Arevalo-Martin et al., 2003; Walter and Stella, 2004). Also, Win 55212-2 was administered daily for 5 days to mice with TMEV either prior to symptoms, at onset of symptoms, or several days after symptom onset (Croxford and Miller, 2003). The results showed that clinical disease symptoms are decreased under all these conditions, while Win 55212-2 increased the susceptibility of mice to TMEV infection, suggesting an immunosuppressive effect, although it had no effect on splenic cell populations (Croxford and Miller, 2003). Win 55212-2 also decreased CNS mRNA encoding for proinflamatory cytokine tumour necrosis factor α (TNFα), interleukin (IL)-1β, and IL-6 in these mice (Croxford and Miller, 2003; Walter and Stella, 2004; Lu et al., 2000). However, in EAE animals there was increased CB_1 receptor activity in the cerebral cortex and caudate-putamen, suggesting that the remaining receptors in these areas may be efficiently coupled to G protein-mediated signalling mechanisms (Berrendero et al., 2001; Walter and Stella 2004).These results show that cannabinoid agonists ameliorate symptoms both acutely and over several weeks in EAE and TMEV models of MS, and the CB_1 receptor expression and function change in EAE, while the absence of CB_1 receptors worsens symptoms of EAE (Simonds, 2003). Although these studies provide insights into the effects of cannabinoids on neuroinflammation, the mechanism of action of these drugs is still incomplete due to differences in the compounds, animal models of MS, rodent species, routes of drug administration, and dosing schedules used (Walter and Stella, 2004).

Microglia have been shown to regulate the initiation and progression of immune responses in the CNS (Carson and Sutcliffe, 1999; Walter and Stella, 2004). Primary cultures of rat and mouse microglia express both CB_1 and CB_2 receptor mRNA and protein (Walter et al., 2003). Human microglia express both CB_1 and CB_2 receptor mRNA, while primary mouse microglia express CB_2 receptors at the leading edges of lamellipodia and micro spikes, suggesting a function in motility (Walter et al., 2003;

Walter and Stella, 2004). The proinflamatory cytokine interferon-gamma (IFN-γ), which is produced by T-helper (T_H) 1 cells and natural killer (NK) cells in MS and EAE, increases CB_2 receptor mRNA and protein in rat microglia (Carlisle et al., 2002).

Astrocytes are the main non-neuronal supporting glial cells in the brain which help to regulate aspects of inflammation in the CNS and may be involved in the pathogenesis of MS. While some evidence of CB_1 receptor expression by astrocytes has been found, it has not been found by all workers (Salio et al., 2002; Walter and Stella, 2004). These conflicting results may indicate variations in CB_1 receptor expression due to differences in species, culture systems, CNS structures from which cultures are derived, ages of cultures, or activation levels of cells, while CB_2 receptor expression by astrocytes has not been found (Walter and Stella 2003; Walter and Stella, 2004). Oligodendrocytes, which undergo degeneration during MS and EAE, also express CB_1 and CB_2 receptors (Walter and Stella, 2004). Some of the major types of glial cells expressing cannabinoid receptors may account for some of the anti-inflammatory effects by cannabinoids in rodent models of MS (Walter and Stella, 2004). It is known that cannabinoid receptor expression is modulated by cytokines in microglial cells but it is not known if cannabinoid receptor expression is modulated in astrocytes or oligodendrocytes (Carlisle et al., 2002; Walter and Stella, 2004).

Neuroinflammation induces a complex and dynamic change in glial cell phenotypes. Microglial cells are one of the first cell types to respond by retracting their processes and migrating towards the site of injury where they release proinflamatory cytokines such as IL-1β, TNFα, and IL-6 (Becher et al., 2000). In primary cultures of mouse microglia, 2-AG induces cell migration and this is reversed by the selective CB_2 antagonist SR144528, cannabinol, and cannabidiol (Walter et al., 2003). This suggests that under neuroinflammatory conditions, neurons or astrocytes produce endocannabinoids as a means of recruiting microglia (Walter et al., 2003; Walter and Stella, 2003).

Nitric oxide (NO) production by glial cells is also associated with immune-mediated cellular cytotoxicity and pathogenesis of MS and EAE (Parkinson et al., 1997). The

cannabinoid agonist CP55940 inhibits NO production in IFN-γ- and lipopolysaccharide (LPS)-stimulated rat microglia (Waksman et al., 1999; Cabral et al., 2001). Primary cultures of rat microglia, when activated by LPS, release TNFα, which is inhibited by cannabinoid agonists: anandamide, 2-AG, Win 55212-2, CP55940, and HU210. However, the antagonists SR141716A, AM251, and SR144528 do not alter the effects of Win 5212-2 on the microglia, suggesting a non $CB_{1/2}$-mediated effects (Facchinetti et al., 2003). Δ^9-THC reduces IL-1β, IL-6, and TNFα production in LPS-stimulated rat microglia (Puffenbarger et al., 2000). The selective CB_2 agonist JWH-015 treatment reduces toxicity of human microglia towards neurons (Klegeris et al., 2003). When these results are compared, they showed that cannabinoid decrease neurotoxicity and release of proinflamatory cytokines from microglia. However it is not known whether these effects are mediated through cannabinoid receptors or other mechanisms (Facchinetti et al., 2003; Klegeris et al., 2003).

Cannabinoids may suppress the immune response and hence the inflammatory response by modulating proliferation or inducing apoptosis in lymphocytes (Malfait et al., 2000). An increase in the number of lymphocytes is crucial for an inflammatory response to occur. The Δ^9-THC induces apoptosis in macrophages (Zhu et al, 1998). Cannabidiol causes a dose-dependent suppression of lymphocyte proliferation (Malfait et al., 2000). Δ^8-THC, CP55940 and anandamide also suppress T- and B- cell proliferation (Schwarz et al., 1994), while CP55940 enhances proliferation of B cells an effect blocked by the antagonist SR144528 (Carayon et al.,1998; Walter and Stella, 2004). Also Δ^9-THC inhibits nitric oxide (NO) production in LPS/IFN-γ-stimulated mouse macrophages and in LPS-stimulated RAW 264.7 macrophages (Coffey et al., 1996; Jeon et al., 1996). Win inhibits the LPS-induced release of NO in macrophages, an effect blocked by the antagonist SR144528 (Ross et al., 2000). The agonist CP55940, reduces NO production from IFN-γ/LPS-stimulated feline macrophages and this is reversed by antagonists SR141716A in CB_1 receptors and SR144528 in CB_2 receptors in both macrophages and primary dorsal root ganglion cells (Ponti et al., 2001; Ross et al., 2000). Plant and synthetic cannabinoids inhibit NO production from immune cells, while the endogenous cannabinoids induce it (Walter and Stella, 2004). Anandamide increases NO production in human monocytes and macrophages (Stefano et al., 1998).

Anandamide stimulates arachidonic acid (AA) release in monocytes and J774 mouse macrophages, an effect blocked by pertussis toxin, an inhibitor of Gi/o proteins (Berdyshev et al., 1997; Di Marzo et al., 1997). Anandamide also induces AA release in cells that do not express CB_1 or CB_2 receptors (Felder et al., 1996; Felder and Glass, 1998). Δ^9-THC induces AA release in RAW 264.7 mouse macrophages and this is likely to be mediated by the CB_2 receptor (Hunter & Burstein, 1997; Felder et al., 2006). The effects of cannabinoids on AA release indicate a proinflamatory influence on peripheral immune cells. It has been shown that cells and tissues involved in neuroinflammation produce and degrade endocannabinoids and anandamide and 2-AG levels are differentially regulated in cells (Walter and Stella, 2004).

1.11. Interaction of opioids and cannabinoids

Opioids and cannabinoids are among the most widely consumed drugs of abuse in the world (Manzanares et al., 1999; Smart and Ogborne, 2000). Both drugs have been shown to share some pharmacological properties including antinociception, hypothermia, sedation, hypotension, inhibition of both intestinal motility and locomotor activity (Manzanares et al., 1999). It has been reported that there is a cross-tolerance or mutual potentiation of these pharmacological effects. These phenomena have supported the possible existence of functional linkage in the mechanisms of action of both drugs especially in antinociception and drug addiction (Manzanares et al., 1999; Manzanares et al., 2005).

The cannabinoid and opioid compounds mimic endogenous ligands and act through the GPCRs, cannabinoid and opioid receptors (Felder and Glass, 1998; Kieffer, 1995). It has been shown that chronic administration of Δ^9-THC increases opioid gene expression while, acute administration of Δ^9-THC increases extracellular levels of endogenous enkephalins in the nucleus accumbens of mice (Corchero et al., 1997; Valverde et al., 2001). Some studies have also demonstrated the existence of cross-tolerance between opioid and cannabinoid agonists and such, morphine-tolerant animals show decreased Δ^9-THC antinociceptive responses, whereas Δ^9-THC-tolerant rodents show a decrease in morphine antinociception (Thorat and Bhargava, 1994; Ghozland et al., 2002). There is cross-dependence between opioid and cannabinoid

compounds and opioid antagonist naloxone precipitated a withdrawal syndrome in Δ^9-THC-tolerant rats, whereas cannabinoid antagonist SR171416A was able to precipitate abstinence in morphine-dependent rats (Navarro et al., 1998; Ghozland et al., 2002). The severity of opioid withdrawal was reduced by the administration of Δ^9-THC or anandamide (Vela et al., 1995; Valverde et al., 2001). This bidirectional cross-dependence was confirmed by using knock-out mice and opioid dependence was reduced in mice lacking the CB_1 receptor whereas, cannabinoid dependence was reduced in mice lacking the preproenkephalin gene (Ledent et al., 1999; Valverde et al., 2000).

Cannabinoids produce their rewarding effects by stimulating mesolimbic dopaminergic transmission which has been shown to be a common substrate for the rewarding effects of other substances of abuse (Tanda et al., 1997). The activation of mu-opioid receptors could be involved in the bidirectional interaction between the endogenous cannabinoid and opioid systems in reward that extends to central mechanisms underlying relapsing phenomena (Fattore et al., 2004). This is because the endogenous cannabinoid system participates in the rewarding effects of opioids (Ghozland et al., 2002), and both morphine self-administration and place preference are decreased in mice lacking the CB_1 receptors (Ledent et al., 1999; Martin et al., 2000). The possible involvement of the endogenous opioid system in the different motivational responses induced by cannabinoids is not yet well understood, however, GABAergic and corticotrophin-releasing factor systems, have been suggested to be involved in the anxiogenic responses induced by cannabinoids and these anxiogenic behaviours could have some influence in the dysphoric effects of cannabinoids (Rodriguez de Fonseca et al., 1996; Ghozland et al., 2002)

Ghozland et al. (2002), have shown that the disruption of mu-, delta-, or kappa-opioid receptor gene does not modify acute Δ^9-THC responses while the expression of Δ^9-THC withdrawal, and the development of Δ^9-THC tolerance is only slightly altered in kappa opioid receptor (KOR) knockout mice. Both mu- and kappa-opioid ligands have been reported to modulate cannabinoid antinociception (Manzanares et al., 1999). The Δ^9-THC antinociception was blocked in mice by the kappa-selective opioid antagonist norbinaltorphimine, and by high doses of the non-selective opioid antagonist naloxone (Smith et al., 1998; Ghozland et al., 2002). The synergistic effects

of morphine and Δ^9-THC on antinociception were also blocked by norbinaltorphimine, a mu selective antagonist (Reche et al., 1996) and high doses of opioid antagonists are usually required to block Δ^9-THC antinociception (Manzanares et al., 1999). Laboratory reports have shown that kappa receptors could contribute to the development of adaptive responses to chronic Δ^9-THC administration, in agreement with the demonstration of cross-tolerance between Δ^9-THC and kappa-opioid agonists (Smith et al., 1994).

A non-selective opioid antagonist naloxone, precipitates an opioid-like withdrawal syndrome in cannabinoid-dependent rodents while, the CB_1 cannabinoid receptor antagonist SR 141716A, induces withdrawal in morphine-dependent rats (Navarro et al., 1998). This suggests that simultaneous activation of the two endogenous systems could participate in both opioid and cannabinoid dependence (Ghozland et al., 2002; Manzanares et al., 2005). Pre-treatment with Δ^9-THC and anandamide, have been shown to decrease morphine withdrawal (Valverde et al., 2001), and the morphine-induced rewarding effects were suppressed in mice deficient in CB_1 cannabinoid receptors, suggesting a bidirectional influence of μ-opioid and CB_1 receptors on reward processes (Ledent et al., 1999; Ghozland et al., 2002).

Ghozland et al. (2002), have proposed that the opposing μ-opioid and κ-opioid receptor activities mediate the dual euphoric-dysphoric effects of Δ^9-THC and a possible mechanism for this could be that cannabinoid receptor activation modifies endogenous opioid peptide levels in mesolimbic areas, that would in turn, modulate dopaminergic activity (Viganò et al., 2005a). The release of opioid peptides by cannabinoids or endocannabinoids by opioids and their interactions at the level of receptor and their signal transduction mechanisms supports the finding of increased opioid peptide levels in the hypothalamus after cannabinoid treatment (Corchero et al., 1997; Viganò et al., 2005a).

Cannabinoids and opioids can also interact at the level of their signalling activities. This is because reports have shown that both cannabinoid and opioid receptor types are coupled to similar intracellular effectors via $G_{i/o}$-proteins, modulating cAMP levels, K^+ and Ca^{2+} channel activities, and MAP kinase phosphorylation (Bouaboula et al., 1995; Fukuda et al., 1996; Manzanares et al., 1999). Vigano et al. (2005a), studied the mechanism of cross-modulation between cannabinoid and opioid systems

for analgesia during acute and chronic exposure. The result showed that acute co-administration of ineffectual sub-analgesic doses of synthetic cannabinoid CP-55,940 and morphine resulted in significant antinociception whereas, in rats made tolerant to CP-55,940, morphine challenge did not produce any analgesic response. The result of Vigano et al., (2005a), study also showed alterations in the cAMP system, which seem to mirror the behavioural responses, indicating that the two systems may interact at the post receptor level which might open-up new therapeutic opportunities for relief of chronic pain through cannabinoid-opioid co-administration.

1.12. B50 Cells

B50 cells are neuronal cell lines derived from the neonatal rat central nervous system (CNS) in 1974 by Schubert et al. (1974). These neuronal cells were cloned using ethyl nitrosourea which form multiple neuronal and glial cell lines, among which are B35 cells, and the B50 cells are the most widely used, while the less widely used ones include B65, B103 and B104 cells. Otey et al. (2003), have shown that of 14 cell lines initially characterised by Schubert and co-workers, two have been in wide use today. These B35 and B50 cells have a near normal karyotype and display normal neuronal properties such as membrane excitability and expression of enzymes for neurotransmitter metabolism, but do not appear to be highly differentiated (Schubert, et al., 1974; Otey et al., 2003; Fashola-Stone, 2005). The B35 and B50 cell lines offer a number of advantages to researchers who study CNS neurons in culture. This is because they are easy to grow, differentiate and transfect.

B50 cells have been used extensively in the study of neuronal cell death, toxicology and differentiation. They have also proved useful in the molecular analysis of endocytosis and of signalling pathways in particular those that guide axonal outgrowth and cell motility (Otey et al., 2003). The B50 cell lines offer substantial advantages over primary CNS neurons for use in cell biological and biochemical experiments, including ease of culture, efficiency of transfection and ability to establish stable cell lines (Reboulleau, 1990; Wu and Ledeen, 1991; Otey et al., 2003). Soon after their development, B50 cells became widely used in the study of factors that control the morphological differentiation of CNS neurons, due to the fact

that they can grow at low density so that neurite outgrowth can be imaged easily in non-overlapping cells, and the rate of neurite extension can be measured unambiguously (Otey et al., 2003).

The B35 cell lines are held by the American Type Culture Collection (ATCC) while the B50 cell lines are held by the European Collection of Cell Cultures (ECACC). Adler (2006b), has shown that the use of cultured cells grown *in vitro* has many advantages over the use of an intact animal or an isolated preparation as an experimental approach. Cultured cells provide a more controlled cellular environment than that achievable in an intact animal. This is because the researcher can selectively alter a single experimental variable and evaluate its effects on a cell with much less concern about possible secondary and tertiary indirect effects from other systems (Adler, 2006b; Adler et al., 2006). Adler (2006b), showed that because cells in culture continue to grow and divide, they can be used to study long-term questions about differentiation, development and the regulation of gene expression.

Cell culture experiments can be divided into two general categories namely those that utilize primary cultures and those that utilize established or secondary cell lines (Adler, 1996). Primary cultures contain cells that have been harvested from an animal or human, dissociated and grow in a culture dish while the established or secondary cell lines are cells that have become adapted to living in culture though they are originally isolated from an animal or a human and have been grown and propagated *in vitro* for many years (Adler, 2006b; Adler et al., 2006; Otey et al., 2003). These secondary cell lines either derived from normal tissues, tumours or transfected with viruses or genes, have been separated from their original tissue sources and have adapted to life outside the body environment where they survive by using nutrients provided in the culture media (Alder, 2006b).

1.13. Aim of the study

The aim of the study was to investigate the effect of hypoxia on neuronal cell signalling and consequent potential neuroprotectant efficacy of cannabinoid and opioid receptor agonists against hypoxia in cortical neuronal B50 cells in culture.

1.14. Objectives and hypotheses of the study

The objectives of the study were to investigate the following:

- The effects of hypoxia on B50 neuronal cell growth and morphology in culture.
- The effects of hypoxia on B50 neuronal cell proliferation, viability and differentiation in culture
- The effects of hypoxia on cannabinoid (CB_1) and opioid (mu) receptors and associated signalling molecules in B50 neuronal cells
- The effects of cannabinoid receptor agonists in the treatment and prevention of hypoxia in neuronal B50 cells in culture
- The effects of opioid receptor agonists in the treatment and prevention of hypoxia on the neuronal B50 cells in culture
- The cannabinoid and opioid agonist-induced effects on the signalling molecules (cAMP and ERK1/2) in cultured B50 cells in hypoxia.

These objectives, when achieved, would enable the testing of the hypotheses of the study that hypoxia adversely affects neuronal cells and cannabinoid, and opioid receptor agonists may have potential therapeutic and protective benefits in hypoxia-induced toxicity in neuronal cells.

CHAPTER TWO

2. Method

2.1 Raising Stable Cells in Culture

2.1.1 Introduction

The cells used in the research were cortical neuronal B50 cell line obtained from the European Collection of Cell Cultures (ECACC) No 85042302. This neuronal cell line was derived from rat central nervous system (CNS) in 1974 and deposited in the tissue culture bank (Schubert et al., 1974). The cells are able to produce action potentials and to extend neurites in culture however, they express higher levels of neurotransmitters and acetylcholine receptors and exhibit a more flattened morphology, suggesting that they represent a more differentiated state than the rest of the cell lines characterised by Schubert and co-workers (Schubert, et al., 1974; Otey et al., 2003; Fashola-Stone, 2005). The B50 cells can be split for sub-culturing at confluency of 70-80% (Fashola-Stone, 2005; ECACC, 2005).

2.1.2 Materials

One ampoule/vial of B50 cells; Dulbecco's Modified Eagle medium (DMEM); Fetal Bovine serum (FBS); Tissue culture Flasks ($25cm^2$ and $75cm^2$); Trypsin-EDTA (0.05% Trypsin, 0.53mM EDTA Liquid); Penicillin-Streptomycin solution; L-Glutamine solution; Dibutyryl cyclic AMP (DbcAMP); Dimethyl sulfoxide (DMSO); Normal Incubator at 37^oC and 5% CO_2; Hypoxic Incubator at 37^oC, 5% CO_2;95% N_2; Pasteur Pipettes; Water bath at 37^oC; 12 well culture plates; Cryogenic Vials; Centrifuge tubes; Micro-centrifuge tubes.

2.1.3 Method

One ampoule containing 1ml of neuronal B50 cells ECACC No 85042302 was ordered from the European Collection of Cell Cultures (ECACC). The cells were resuscitated by warming-up the cells to room temperature for one minute until they were fully thawed. The thawing process was carried out quickly to avoid damage to the cell membrane (ECACC, 2005). The ampoule was then wiped with tissue soaked with 70% alcohol before opening to prevent cross-contamination of cells. The cells were slowly pipetted into a pre-warmed culture media in a $25cm^2$ culture flask and

incubated in the normal incubator at 37^{o}C and 5% CO_2. The culture media was made-up of the following components:
Dulbecco's Modified Eagle's Medium (DMEM), 10% Foetal Bovine Serum (FBS) (Inactivated), 1% Penicillin-Streptomycin solution (100U/ml) and 2mM L-Glutamine.

The method of inactivation of the foetal bovine serum (FBS) was by the thermal method in which the FBS was heated in a hot water bath at 56-60^{o}C for 90 minutes and allowed to cool before being mixed in the appropriate proportion with the Dulbecco's modified eagle's medium, penicillin-streptomycin and L-glutamine solutions to make up the working solution of the culture media.

The cells were stored in the normal incubator and were observed under the microscope every 24 hours of incubation to monitor their growth and wellbeing in culture. At about 80 to 95% confluency level since the recommended confluency for B50 cells sub-culturing is between 70 to 80% (Fashola-Stone, 2005; ECACC, 2005), the culture medium was decanted. Since the B50 neuronal cells are adherent, they were treated with 0.25% solution of Trypsin-EDTA at 2ml to the 25cm^2 culture flask to ensure that all the cells were covered. The cells were incubated for approximately 2 minutes at 37^{o}C to enable all the cells to be removed from the bottom of the flask. At the end of the incubation period, a fresh culture medium was added which stops the activity of the Trypsin-EDTA solution and then resuspend the cells. The cells were then centrifuged at a speed of 2500 x g for 5 minutes. After the centrifugation, the medium was decanted and a fresh medium added to the cells. The cells were split into new culture flasks in 1:4 ratios with fresh media for sub-culturing while some of the cells were frozen at -70^{o}C to enable them to be preserved for future use.

2.1.4 Method for Freezing B50 Cells

The freezing media was prepared using 90% foetal bovine serum (FBS) and 10% cryoprotectant dimethylsulphoxide (DMSO). The cells were resuspended in the freezing medium and then aliquoted at 1ml per cryogenic ampoule. The ampoules were wrapped in cotton wool, placed in a box container and frozen at -70^{o}C. After 24 hours the cells were removed from the box and restored at -70^{o}C. The cells were tested for viability by thawing one ampoule and starting a new culture with it.

2.2 Total cell count and viability

2.2.1 Introduction

A cell and viability count is a determination of living or dead cells based on a total cell sample (Ellis-Christensen, 2007). Cell viability measurements are used to evaluate the number of live or dead cells in experimental conditions. The measurement of cell viability involves looking at a sample cell population and staining the cells, or applying a dye, to show which cells are living and which ones are dead under the microscope (Yamauchi et al., 2003). The objective of this study was to determine the total cell count (cell concentration), total number of living cells (viable) and dead cells (non-viable) and the percentage of living cells (%viable cells), in B50 cells cultured under normal and hypoxic incubators, using the trypan blue exclusion method. In this method, the dead or non-viable cells take up the dye, while the viable or living cells do not (Li and Gong, 2007).

2.2.2. Materials

12-well culture plates; Culture media; Olympus microscope; Trypan blue (0.4%); Haemocytometer; Pasteur pipettes and Cover slips.

2.2.3. Method

The cells were cultured in 12-well culture plates in both normal and hypoxic incubators. The cells were harvested after 24, 48, 72, 96, 120 and 144 hours of culture, while the 0 hour was regarded as the starting point of the culture. The cells were harvested by detaching them from the plates using a 0.25% solution of Trypsin-EDTA and centrifuged at 2500 x g for 5 minutes. The supernatants were decanted and cellular pellets collected. The cells were resuspended in the culture medium and studied using trypan blue exclusion method. This method involved pipetting 50µl of cell suspension into a tube with 50µl of the 0.4% (w/v) solution of trypan blue dye and left to stand at room temperature for 5 minutes. The haemocytometer was fixed with a cover-slip and 20µl of the cell suspension/trypan blue mixture was applied between the coverslip and the haemocytometer. The haemocytometer chambers were filled by capillary action and the cells were viewed under the microscope (magnification x 400) to show a single counting square (Figure 2.1). With the microscope focused on the gridlines of the chamber, the viable cells (those that did

not take-up the dye) were not coloured, while the non-viable cells (those that absorbed the dye) were coloured blue (Yamauchi et al., 2003; Li and Gong, 2007) . The cells were counted in four corner squares of one chamber as shown in Figure 2.2. The cells that lie on the lines were not counted unless they were touching the top and left-hand lines of each corner square, in accordance with the manufacturer's instruction. At each session of the experiment, three independent samples were randomly selected from different plates and each experiment was repeated two times to give the number of samples (n = 6) for measurement of viability and cell death, and was used to obtain the mean and standard deviation. Viability of culture was calculated as the percentage of the ratio of the number of unstained cells (viable cells) to the total number of cells counted (viable cells plus nonviable cells). The total number of cells, total viable cells, and percentage viability were calculated using the formulae below:

Total cell count/ml = (number of viable cells counted + number of non-viable cells counted) x10^4 x dilution factor

$$\text{Viable cell number/ml} = \frac{\text{(number of viable cells counted)}}{\text{number of squares counted}} \times 10^4 \times \text{dilution factor}$$

$$\text{Percentage Viability} = \frac{\text{(number of viable cells counted)}}{\text{Total cell count}} \times 100$$

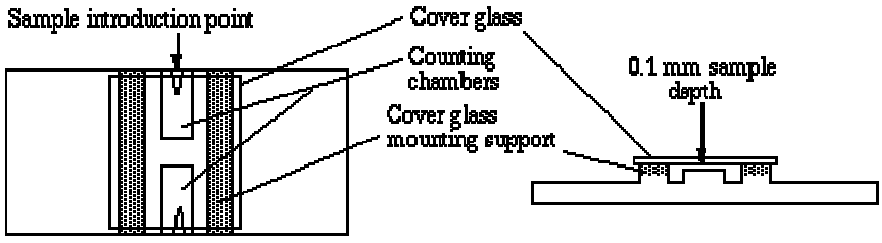


Fig.2.1. Representation of the counting chambers of haemocytometer.

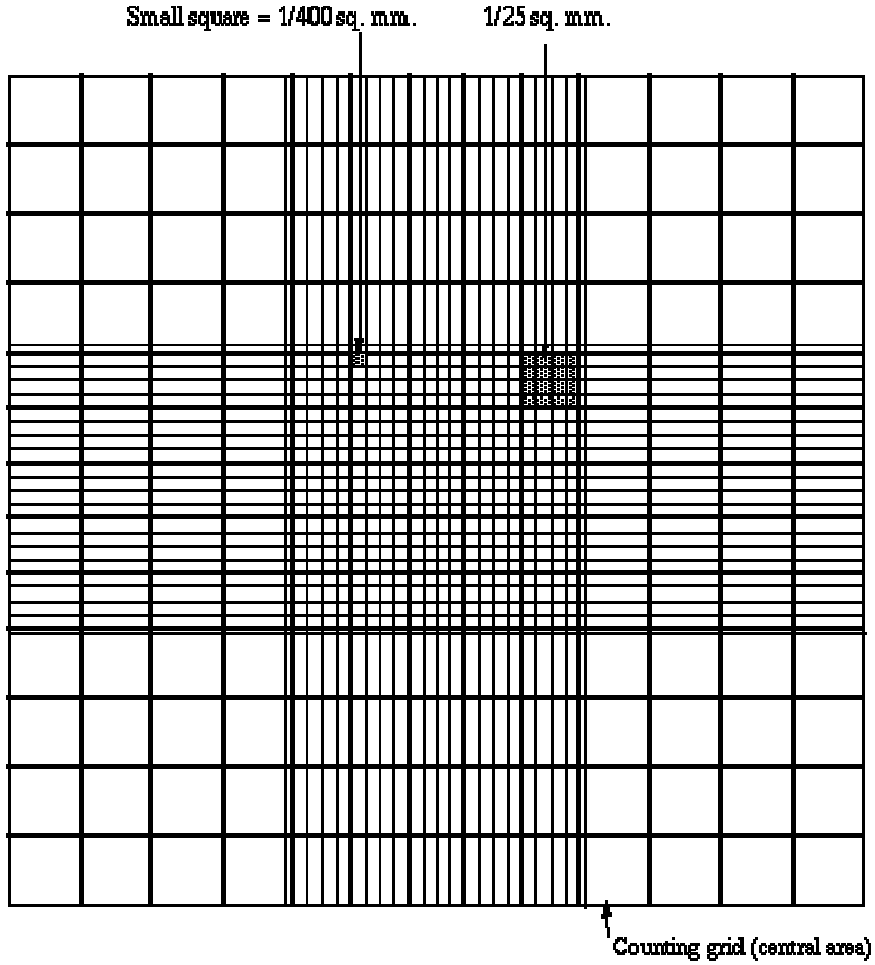


Fig 2.2 Representation of the haemocytometer counting squares.

2.3 The effect of hypoxia on B50 neuronal cells in culture using LDH assay

2.3.1 Introduction

Lactate dehydrogenase (LDH) release has been shown to be a reliable marker and index of cellular damage (Zhang et al., 2002; Zhang et al., 2005; Ma et al., 2005; Zhang et al., 2006). The LDH assay measures cytotoxicity and cell lysis by detecting the LDH activity released from damaged and injured cells (Zhang et al., 2006; Ma et al., 2005). The LDH assay can be used in many different *in vitro* cell systems where cellular damage, lysis or injury of the plasma membrane may occur (Andre et al., 2004; Laughton et al., 2007). The assay can also be used to quantify the number of viable cells and cellular growth in neuronal cultures (Aoki et al., 2007; Tweedie et al., 2006). The LDH assay is simple, accurate and yields reproducible results (Decker and

Lohmann-Matthes, 1988; Weidmann et al., 1995; Sigma, 2006; Arthur et al., 2007). This assay method is simple and convenient because all the assay components are included in one complete assay kit and could be measured using different types of microplate reader. It is accurate and reliable because it has long been used as a measure of cellular death and viability in hospitals as a marker for myocardial infarction (Elikowski et al., 2006), but was later introduced as a measure of cellular viability and death in neuronal cultures (Zhang et al., 2006; Li et al., 2005; Tateno et al., 2008). The assay is based on the reduction of NAD^+ by the action of the LDH enzyme and the resulting reduced NAD^+ (NADH) is utilised in the conversion of tetrazolium dye, resulting in a coloured compound, formazan that is measured spectrophotometrically (Sigma, 2006; Carr et al., 2005; Legrand et al., 1993; Abe and Matsuki, 2000). The amount of formazan that is actually measured correlates directly to the amount of the LDH released by the injured, damaged or lysed cells in the culture (Tian et al., 2007; Xu et al., 2007). The LDH assay has been shown to be sensitive, convenient and precise and is applicable to a variety of toxicity studies (Abe and Matsuki, 2000; BioVision, 2006; Guzman, 2005). The aim of the study was to investigate the effect of hypoxia on LDH release from cultured B50 neuronal cells.

2.3.2 Materials

Lactate Dehydrogenase Assay Kit (Sigma Tox-7), containing: LDH Assay Substrate Solution (L2402); LDH Assay Cofactor Preparation (L2527); LDH Assay Dye Solution (L2277) and LDH Assay Lysis Solution (L2152); 1N Hydrochloric acid (H9892); 12 well culture plates; culture media; Trypsin-EDTA solution; Centrifuge and Centrifuge tubes; Normal and hypoxic Incubators; 96 well Plates; Dynex MRX Micro plate Reader at 490 nm.

2.3.3 Method

The B50 neuronal cells were allowed to grow in 12-well culture plates for 96hrs after resuscitation in both normal and hypoxic incubators. The cells with the culture plates were removed from the incubators into the laminar flow hood and centrifuged at 250 x g for 4 minutes to pellet cells. Aliquots of the supernatants were transferred into a clean flat bottom 96 well micro plate.

LDH assay working solution

The working solution of LDH assay cofactor was prepared by adding 25ml of deionized, sterilized tissue culture water to the bottle of lyophilized cofactors. The lactate dehydrogenase assay mixture was prepared by mixing equal amounts (20ml) of LDH assay substrate, LDH assay cofactor and LDH assay dye solution. The LDH assay mixture was added at two times (40µl) the volume of the supernatant medium (20µl) removed for assaying. The plates were covered with aluminium foil to protect them from light and incubated at room temperature for 30 minutes. The reaction was terminated by the addition of one tenth volume (6µl) of 1N HCL solution to each well. The experiment was done in triplicate for two times (n = 6) according to the method of Bouaboula et al. (1995). The absorbance was spectrophotometrically measured at a wavelength of 490nm using a Dynex MRX model of micro plate reader. The result was calculated by using the formula below:

1) Total Absorbance = Sum of (n) Absorbances (n = 6)

2) Mean Absorbance = $\frac{\text{Total Absorbance}}{\text{Number of Absorbance (n)}}$

3) Mean Corrected Absorbance = (Mean test Absorbance – Mean Background Absorbance)

4) Percentage (%) absorbance = $\frac{\text{Mean Test Absorbance}}{\text{Mean Control Absorbance}} \times 100$

Background Absorbance = Absorbance from the culture medium only

Control Absorbance = Absorbance from the normal cells with medium

Corrected Absorbance = corrected Absorbance without background

Test absorbance = Absorbance from the experimental cells with the culture medium

2.4. The effect of culture duration on LDH release from B50 cells in normal and hypoxic cultures

2.4.1 Introduction

The duration of cultures have been shown to influence and affect the amount and level of cellular damage, lysis and finally death in both normal and experimental conditions (Ying et al., 2003; McInnis et al., 2002). This study was designed to evaluate the influence of time duration of B50 cells in culture on LDH release in both normal and hypoxic cultures.

2.4.2 Materials

12 well culture plates; Centrifuge and centrifuge tubes; Culture media; LDH kit; Normal Incubator and hypoxic Incubator.

2.4.3 Method

The B50 cells were cultured normally in 25cm^2 culture flasks and split into 12 well culture plates. The cells were cultured in both normal and hypoxic conditions at different time durations (24, 48, 72, 96, 120 and 144 hours). At the end of each time interval using 0 hr as the starting point of culture, the cells were harvested and assayed for LDH release from both the normal and hypoxic groups of cells. The results were calculated as in session 2.3 above and compared between the groups of normal and hypoxic cells.

2.5. Neuronal cell proliferation

2.5.1 Introduction

Different types of cells have different levels of metabolic activity. It has been shown that factors that affect the metabolic activity of cells may affect their viability and consequently their proliferation (Ellis-Christensen, 2007; Kumar, 2004). Dhanasekaran et al. (2004) have shown that the alpha-subunits and the beta-gamma-subunits of G proteins regulate several critical metabolic and signalling pathways involved in cell proliferation, differentiation and apoptosis. These pathways include the mitogen activated protein kinase (MAPK), cyclic adenosine monophosphate (cAMP) and cAMP response element-binding (CREB) protein (Barco et al., 2006).

Adherent cells that undergo contact inhibition may show a change in metabolic activity per cell at high densities, resulting in a non-linear relationship between cell number and cell proliferation when using assays involving metabolic function to assess proliferation (Promega, 2006; Gibbs, 2003). Cells undergoing contact inhibition especially at 100% confluency in culture result in arrest of further cell proliferation and growth due to lack of culture space (Morgan, 2007). It has been shown that factors which affect the physiology of the cells or their cytoplasmic volume will affect their metabolic activities and hence their proliferation and viability (Zhang et al., 2002). Hence cell proliferation and viability assessment was measured by the use of CellTiter 96 AQueous One Solution Assay (Promega). This assay method is a non-radioactive procedure that measures metabolic function and directly correlates it with living cell numbers (Roehm et al., 1991; Goodwin et al., 1995). The aim of this work was to study the effect of hypoxia on neuronal B50 cell proliferation and viability using the Promega CellTiter one solution method.

2.5.2. Materials

12 well culture plates; Culture media; Normal and hypoxic incubators; Promega Cell Titer One solution cell proliferation assay kit; Dynex MRX Model 96 well micro plate reader at 490nm; Centrifuge tubes and 96 well plates.

Cell Titer 96® One solution

The Cell Titer 96® One Solution cell proliferation assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxic assays (Promega, 2006; Goodwin et al., 1995). The reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyoxyphenyl)2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; [MTS] and an electron coupling reagent, Phenazine ethosulphate (PES) (Roehm et al., 1991; Promega, 2006). PES has an enhanced chemical stability, which allows it to be combined with MTS to form a stable solution (Roehm et al., 1991). The MTS tetrazolium compound, Owen's reagent is bio-reduced by cells to form a coloured formazan product that is soluble in tissue culture medium (Roehm et al., 1991; Promega, 2006). This conversion is accomplished by the NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (Goodwin et al., 1995; Zhang, et al., 2002).

2.5.3 Method

The neuronal B50 cells were cultured in 12 well culture plates in both normal and hypoxic incubators for time intervals of 24hrs, 48hrs, 72hrs, 96hrs, 120hrs and 144hrs prior to being harvested. The Cell Titer 96® one solution reagent was obtained from Promega and stored frozen at -70°C and thawed at time of use to room temperature. Since the B50 cells are adherent cells, 0.25% Trypsin-EDTA was used to detach the cells from the plate and at the end of the trypsinization process, the culture medium was used to stop the action of the trypsin-EDTA solution. The cell solutions were centrifuged at 2500 x g for 5 minutes and the supernatants were removed leaving the cells. The cells were collected and resuspended with the culture media. 100µl of the culture media containing the resuspended cells, were pipetted into each well of the 96-well assay plate and 20µl of the Cell Titer 96® One Solution reagent added. The solutions were incubated for 2 hours at 37°C in a humidified 5% CO_2 chamber. The absorbance was recorded at 490nm using the Dynex MRX Model 96 well micro-plate reader. Each experiment was performed in triplicate and repeated two times (n = 6). The absorbance values were corrected by subtracting the mean absorbance of the groups from the absorbance of the wells containing only culture medium with no cells (background) and were used to determine the number of viable cells in proliferation from the normal and hypoxic cultures of B50 cells. Since it has been shown that cellular viability and proliferation are directly proportional to the absorbance measured (Xiong et al., 2007; Chett et al., 2007), the absorbance can be used as an index of cellular proliferation and viability. The results were calculated as follows.

a) Mean Absorbance = $\frac{\text{Sum of absorbances}}{\text{Number of assays (n= 6)}}$

b) Mean Test Absorbance= [Absorbance of cells $_{(\text{Tests})}$–Absorbance of medium $_{(\text{Background})}$]

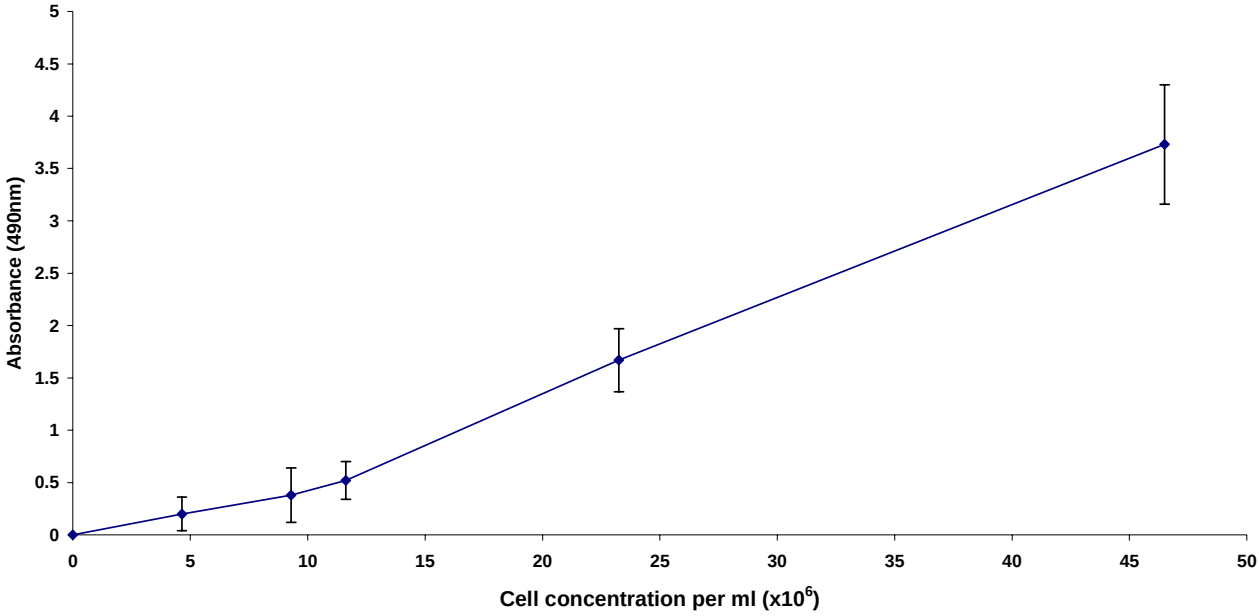
c) A standard curve for B50 cells was prepared using trypan blue cell counting method. A culture plate containing 46.5 x 10^6cells/ml was used to prepared five dilutions (0, 2, 4, 5 and 10) x 10^6cells/ml. Cells were transferred into the microplate, treated with CellTiter Aqueous One Solution and absorbance (n=5) was measured spectrophotometrically at 490nm. The absorbance was plotted against the known number of cells to give a standard curve (Figure 2.3). The number of cells from the

test groups was derived by plotting the absorbance of the test groups against the known cell numbers from the standard.

d) The rate of proliferation was calculated by dividing the cell numbers by time (24hrs) to give the daily proliferation rate.

$$\text{Proliferation rate} = \frac{\text{Number of cells per ml}}{\text{Time (24 Hours)}}$$

Fig.2.3.Standard curve of the time-course effect of hypoxia on B50 cell absorbance of the CellTiter One solution proliferation assay at 490nm as a function of cell concentration



2.6. Morphological Studies

2.6.1 Introduction

The study of the morphological changes in B50 neuronal cells is necessary to ascertain the level of their activity and functionality. This is because the morphology of cells indicates the status of the cells, both in terms of the health of the cells and their physiological activities. Changes in the physiological processes and activities in cells are often accompanied by changes in cellular morphology. Examples include changes in the intracellular location, arrangement and structure of cellular constituents (such as organelles, macromolecular clusters or the cytoskeleton), changes in the morphology of the entire cell (such as its shape and area), changes in the spacing and proximity between cells, and properties of multi-cellular colonies (such as its shape, size and cell locations) (Yamamoto et al., 2001). It has been shown that normal morphology is concomitant to normal physiology, while abnormal morphology is synonymous to altered function which is mostly observed in the pathological changes that occur in different organs and tissues of the body (Lobner et al., 2000). If the morphology of B50 cells is normal that gives an indication of their normal signalling function and activities. The aim was to study the changes that may occur in the morphology of neuronal B50 cells in normal and hypoxic conditions.

2.6.2 Materials

12 well culture plates; 25cm^2 culture flasks; Culture media; Microscope and peripheral accessories.

2.6.3 Method

The frozen B50 cells were thawed and revived into active cell cultures. The cells were raised in culture for 24, 48, 72, 96, 120 and 144 hours while 0 hour was regarded as the starting point of culture during splitting of cells for sub-culturing. At each stage of the experimental period, the cells were observed under the microscope, using same field morphological assessment in which the culture plates were examined from the centre to the sides in a quadri-point analysis method (Ellingson et al., 2007; Sato and Momose-Sato, 2007), and any change in the morphology of the cells was noted. This was repeated three times for each experiment. Micrographs of the cells were taken at the different time intervals at a magnification of 200 times (x 200), to show the

morphological changes that may have occurred between the normal and hypoxic cells in culture.

2.7 Neuronal B50 cell differentiation

2.7.1 Introduction

Neuronal functions and activities depend on their normal differentiation. Neuronal differentiation makes it possible for neurons to differentiate into the cell body containing the nucleus and cytoplasm, a single axon and different numbers of dendrites (Yoshimura et al., 2006). This neuronal differentiation makes it possible for nerve cells to receive neural signals from the dendrites, integrate them in the cell body and finally send these signals out to other neighbouring neural cells through the axon into the synapses. If these neurons are undifferentiated or not properly differentiated, the signals cannot be normally received and transmitted through the dendrites, cell body and axons since the primary function of the nerve cells is to receive, integrate and transmit signals (Yoshimura et al., 2006). Dibutyryl cyclic adenosine monophosphate (DbcAMP), has been shown to induce neuronal cell differentiation in culture (Park et al.,1998; Fahrig and Sommermeyer, 1993). DbcAMP has been shown to be an inducer of cellular morphological differentiation in culture in varying concentrations ranging from 0.5mM, 1.0mM, 1.5mM and 2.0mM depending on the type of cell used (Boghaert et al., 1991; Nagamine et al., 1990; Shimizu et al., 1994). It has been shown that neuron-type cells undergo cellular differentiation when treated with 1mM concentration of DbcAMP (Oda et al., 2007; Hu et al., 2007). On addition of DbcAMP to cells grown in culture, cell division is arrested and the cells rapidly undergo distinctive morphological transformation characterized by neurite extension and reduction in saturation densities (Siegel and Lukas, 1988; Oda et al., 2007). The aim of this experiment was to study the effect of hypoxia on the neuronal B50 cells differentiation in culture using DbcAMP.

2.7.2. Materials

Dibutyryl cyclic adenosine monophosphate (DbcAMP); Culture media; 12 well culture plate; Microscope.

2.7.3 Method

The neuronal B50 cells were cultured in both normal and hypoxic incubators. The B50 cells were stimulated to differentiate by making up the culture media with 1mM DbcAMP. The level of neuronal differentiation was assessed microscopically, after 96hrs of culture in both normal and hypoxic cultures. The B50 cells were assessed by observing the appearance of the neurites specifically the axons and dendrites called the neural processes (Fahrig and Sommermeyer, 1993). The third group of cells are those that were cultured for 48hrs in a normal incubator and then transferred into the hypoxic incubator for a further 48hrs followed by a microscopic assessment of the level of cellular differentiation. The level of the effect was studied by random field strip counting of the number of differentiated B50 cells, under the microscope at a magnification of 200 times (x 200), for the three groups. The differentiated cells counted contained two neuritic processes (axon and dendrite), which were longer than cell body diameter according to the method of Richter-Landsberg and Jastorff (1986) and Oda et al. (2007). Each count was repeated two times and all the experiments were performed three times (n = 6).

2.8 Neuronal pattern and pattern formation

2.8.1 Introduction

Neuronal pattern and pattern formation makes it possible for neurons to organise themselves into groups called nerve fibres that carry neural signals into specific areas of the body to and from the brain. These patterns and the pattern formation itself are very important in ensuring that specific signals are carried to specific areas for the effective and efficient coordinated neural response (Golubitsky et al., 2004). The neuronal fibres are arranged in bundles that receive and transmit related signals. These bundles form a neural tract e.g. the visual tract carries visual signals from the eyes to relay centres in the brain and back to the eyes for visual perception to occur (Cowan and Thomas, 2004). Asare et al. (1996), have shown that spatial difference exist in the distributional pattern of neurons in the superior frontal gyrus of 32 subjects who died of acquired immune deficiency syndrome. This gives support to the fact that, if there is a disruption in the tracts, the signal may not be properly

coordinated and relayed for the perfect response to occur. Genetic coding has been shown to play a role in the pattern formation in various part of the nervous system (Schmid et al., 2000a). The aim of this experiment was to study the effect of hypoxia on neural pattern and pattern formation in B50 neuronal cells in culture.

2.8.2. Materials

$25cm^2$ culture flask; 12 well culture plates; culture media with and without serum; Microscope; normal and hypoxic Incubators.

2.8.3 Method

The B50 neuronal cells were cultured in both normal and hypoxic incubators for 48 hrs with normal culture media containing 10% Foetal bovine serum (FBS). The culture media was decanted after the first 48hrs and another culture media, without FBS (serum-free culture medium), was added to the cells for 72 hours to induce neuronal differentiation and hence pattern formation (Martín-Orozco et al., 2007). It has been shown that cellular differentiation is induced in a serum-free culture media (Gu et al., 2007; Nam et al., 2007). After 120 hrs of culture, the cells were observed under the Nikon Eclipse TS100 microscope using same field morphological assessment in which the culture plates were examined from the centre to the sides in a quadri-point method (Ellingson et al., 2007) at a magnification of 200 times. Each experiment was repeated three times and micrographs were taken using an IBM computer based Image Solutions® to analyse the changes that may have occurred between the normal and hypoxic cells in culture.

2.9 Cannabinoid (CB_1) and Mu opioid receptor expression in neuronal B50 cells using semi-quantitative RT-PCR Method

2.9.1 Introduction

Reverse transcription (RT) polymerase chain reaction (PCR), is a laboratory method used for amplifying a piece of ribonucleic acid (RNA) molecule (Prasad et al., 2001). The RNA strand is first reverse-transcribed into its deoxyribonucleic acid (DNA) complement or complementary DNA, followed by amplification of the resulting DNA

using a polymerase chain reaction (Prasad et al., 2001). The RT-PCR based assay is one of the most common methods for characterizing and confirming gene expression patterns using messenger ribonucleic acid (mRNA) in different sample populations (Orlando et al., 1998; Bustin, 2002). The amplification of RNA using RT-PCR provides for a highly sensitive technique, where a very low copy number of RNA molecules can be detected and is widely used in the diagnosis of genetic diseases and, quantitatively, in the determination of the specific RNA molecules within a cell or tissue as a measure of gene expression (Nolan et al., 2006; Burdyga et al., 2004). Bustin (2002), has shown that accurate determination of total RNA concentration is important for the quantification of mRNA levels and any DNA contamination will result in inaccurate quantification. The RT is important for sensitive and accurate quantification and the amount of cDNA produced by the reverse transcriptase accurately represents the RNA input (Gnanapavan et al., 2002). It has been shown that the range, sensitivity and specificity of the enzyme are important considerations for a successful RT-PCR assay (Bustin, 2002; Wall and Edwards, 2002; Liu et al., 2002). The RT-PCR quantification of mRNA has been used to monitor transcription *in vitro* and direct detection of the effects of receptor signalling (Liu et al., 2002; Yuen et al., 2002; Cohen et al., 2002). The RT-PCR method has also been used to study the effects of some experimental agents on the expression of cannabinoid and opioid receptors in whole animal and culture conditions (Lalonde et al., 2006; Chen et al., 2005; Zhang et al., 2006). The aims of the experiment were to study the following:

- to investigate the expression, and hence the presence, of cannabinoid (CB_1) and mu opioid receptor mRNA in B50 neuronal cells.
- to investigate the effect of hypoxia on the expression of cannabinoid (CB_1)and mu opioid receptor mRNA on B50 neuronal cells
- to investigate the effect of cannabinoid and opioid receptor agonist treatment on cannabinoid (CB_1) and mu opioid receptor mRNA in cultured B50 cells using semi quantitative RT-PCR.

2.9.2 Extraction of total RNA

Introduction

The B50 cells were cultured and exposed to different experimental conditions. The total cellular RNA was extracted from the cultured B50 neuronal cells using the TRIzol reagent method (Invitrogen No 15596-026), as outlined below.

Materials

Cultured B50 cells in different experimental conditions; Chloroform; Isopropyl alcohol; 75% Ethanol in DEPC-treated Water; RNase-free water; Diethylpyro carbonate (DEPC); TRIzol reagent (Invitrogen No 15596-026); RNaseZAP (Sigma R2020), used in washing all the wares to eliminate RNA contamination; Micro centrifuge tubes and Centrifuge.

Method

The B50 cells in different experimental conditions were grown and lysed in culture plates by adding 0.5ml of TRIzol reagent to each well. The cells were homogenized and incubated for 5mins at room temperature. The homogenates were transferred to micro-centrifuge tubes, 0.1ml of chloroform added, the cap secured and the tubes shaken vigorously by hand for 15 seconds. The cellular mixture was incubated at room temperature for 3 minutes. The mixture was then centrifuged at 12,000 x g for 15 minutes at room temperature. Following centrifugation, the mixture was separated into 3-layers namely a lower phenol-chloroform phase (Red), a middle interphase (Cloudy) and an upper aqueous colourless phase.

The RNA is present at the upper aqueous colourless phase and formed about 60% of the total volume of the mixture and was transferred to a fresh micro-centrifuge tube. The aqueous phase was mixed with 0.25ml of isopropyl alcohol and incubated for 10 minutes at room temperature. The mixture was then centrifuged at 12,000 x g for 10 minutes at room temperature. At this point the RNA precipitated and formed a gel-like pellet.

The supernatant was removed and the remaining RNA pellet was washed once with 0.5ml of 75% ethanol and mixed by vortexing. The mixture was centrifuged at 7,500 x g for 5 minutes at room temperature and the ethanol was decanted. The RNA was

then air dried for 10 minutes, dissolved in 100% deionized formamide and stored at -70^{o}C to be used in RT-PCR analysis.

2.9.3. Semi-Quantitative One step RT-PCR Analysis

Introduction

The semi-quantitative one step reverse transcriptase polymerase chain reaction (RT-PCR) was used to study the expression of cannabinoid and opioid receptors. The method used was the Superscript III One-Step RT-PCR system with Platinum Taq DNA polymerase from Invitrogen (12574-018). This method is a sensitive, reproducible, end point detection and analysis of RNA molecules by RT-PCR (Chen et al., 2005; Invitrogen, 2005). This method offers a convenient One-Step formulation which can be used to perform both complementary DNA (cDNA) synthesis and PCR amplification in a single tube using gene specific primers and target RNAs from either total RNA or mRNA (Prasad et al., 2001; Invitrogen, 2005). The system uses a mixture of superscript III Reverse Transcriptase and Platinum Taq DNA polymerase in an optimized reaction buffer and can detect a wide range of RNA targets, while the amount of starting material can range from 0.01pg to 1.0 µg of total RNA (Invitrogen, 2005).

Cannabinoid (CB_1) and mu opioid receptors (MOR) were selected for the semi-quantitative RT-PCR analysis because the CB_1 receptors are predominantly found in the neurons of the CNS and the B50 neuronal cells are derived from the CNS neurons. MOR were selected for study because opioid receptor subtypes have been shown to have 68% sequence homology between them and the differences between them was proposed largely, on the basis of radioligand binding studies and as such there is little or no evidence for the presence of the different genes encoding the opioid receptor subtypes (Corbett, et al., 2006). In some cases receptor heterodimerisation of opioid receptors has been proposed as a possible explanation for the different opioid receptor subtypes (Corbett, et al., 2006; Milligan, 2004).

Materials

The extracted total RNA, Superscript III RT/Platinum Taq Mix, Reaction Mix, 5nM Magnesium Sulphate, GeneAMP PCR System thermal cycler. CB_1 primers, sense 5'-GAT GTC TTG GGA AGA TGA ACA AGC-3'(nt 365-373) and antisense 5'-AGA CGT GTC TGT GGA CAC AGA CAT GG-3'(nt 460-468). The primers were selected from Esposito et al. (2002), since they were used for the study of CB_1 expression and the effect of cannabinoid agonist treatment in neuronal glial cells. Mu opioid receptor (MOR) sense primers**,** 5-GGA ACA TGG CCC TTC GGA ACC ATC-3' (574-597) and antisense 5'-TAC CAG GTT GGG TGG GAG AAC GTG-3' (863-840), were selected from Silbert et al. (2003), where they were used to study the effect of MOR expression and opioid treatment in myelinated and unmyelinated neurons. Alpha actin primer Sense 5'-GAT CAC CAT CGG GAA TGA ACG C-3' (389bp) and Antisense 5'-CTT AGA AGC ATT TGC GGT GGA C-3', selected from Park et al., (1997), where they were used as an internal control for cytoskeletonal study in pericytes.

Method

Programming of the thermal cycler

- cDNA synthesis 1 cycle at 55^{o}C for 30 minutes
- Denaturation 1 cycle at 94^{o}C for 2 minutes
- PCR amplification 40 cycle at 94^{o}C for 15 seconds (Denature)
  60^{o}C for 30 seconds (Anneal)
  68^{o}C for 60 seconds (Extend)
- Final extension 1 cycle at 68^{o}C for 5 minutes

Preparation of Master Mix

The master mix was prepared on ice using 0.2ml nuclease free, thin walled PCR tubes. Each PCR tube contained the following:

- 2 x Reaction mix (dNTPs:200µM; $MgSO_4$:1.6mM) 25µl
- Template RNA (200ng/µl) 1µl
- Sense Primer (0.2µM) 1µl
- Antisense Primer (0.2µM) 1µl

- SuperScript III RT/ Platinum Taq mix (5mM) 2µl
- Autoclaved distilled water; 20µl was added to make up total volume to 50µl

These were mixed together gently and all the components were allowed to settle at the bottom of the amplification tube. The tubes were then centrifuged briefly by pulse centrifugation, over-laid with one drop of mineral oil and placed in a preheated thermal cycler (GeneAMP PCR System) as programmed above. One tube was used as blank and contained only master mix and water.

Analysis of the RT-PCR products

The analysis of the PCR products was carried out using the following method. Agarose gel (2%) was made by dissolving 2.5g agarose in 112.5ml of distilled water which was subsequently micro-waved in a conical flask for 5 minutes. 12.5ml buffer solution of Tris/Borate/EDTA (TBE) was added and 5µl of ethidium bromide added for staining the mRNA and mixed thoroughly. The mixture was poured into the gel tray with combs in place and air bubbles pushed to the sides with a pipette. The gel was allowed one hour to set. 5µl of Blue loading buffer (Sigma, G7654), which contain bromphenol blue (0.25% w/v), xylene cyanole FF (0.25% w/v), sucrose (40% w/v), was added to each of the samples and centrifuged briefly to mix and settle.

A 10µl DNA ladder (Promega, G2101) and 5µl Blue loading buffer was used to makeup the DNA marker, and one litre of gel buffer was made using the TBE in a 1:10 dilution. The gel was placed in an electrophoresis tank with wells at the negative electrode such that RNA will move towards the positive electrode. The gel was then surrounded with gel buffer and the wells completely covered by the buffer. The DNA marker (5µl), was added to the gel on position one, followed by adding a 10µl blank sample to the next position, and continuing to add 10µl of each sample to the remaining wells. The electrophoresis tank was connected to a power supply set at 125V for one hour. Photographs of the gels were taken and scanned using the digital densitometer to evaluate and semi-quantify the mRNA of the receptors, and then compared between the different groups.

2.10. The effect of cannabinoid receptor agonists on B50 neuronal cell morphology, proliferation and viability.

2.10.1 The effect on morphology

Introduction

Cannabinoid receptors of the CB_1 type mediate the central nervous system actions and they belong to the super family of seven transmembrane G-protein coupled receptors (Breivogel et al., 1997; Griffins et al., 1998; Breivogel et al., 2004). Cannabinoids have been shown to protect neurons from death caused by glutamatergic over stimulation, ischaemia and oxidative damage (Chen et al., 2005; Hansen et al., 2002; Guzman et al., 2002; van der Stelt and Di Marzo 2005). The aim of this study was to evaluate the effects of three different synthetic cannabinoid receptor agonists on the morphology of B50 neuronal cells cultured under hypoxic conditions.

Materials

Three different synthetic cannabinoid agonists from Tocris, namely: Win 55,212-2 mesylate (Win); anandamide or arachidonylethanolamide (AEA); and 2-arachidonyl glycerol (2-AG); normal and hypoxic incubators, culture media and 12 well culture plates.

Method

The cells were defrosted and resuscitated in culture plates for 24hrs and the three synthetic cannabinoid agonists (Win; AEA and 2-AG) were dissolved separately in 2% DMSO (Sigma) to form 5mM stock solution of the drugs. The three agonists were selected because of their activation of the CB_1 receptors which are found mostly in the CNS neurons and each agonist was selected because: 1) Win is a potent non-selective CB_1/CB_2 cannabinoid receptor agonist, with higher affinity for the CB_1 receptors in the CNS when compared to CB_2 receptors (Ferraro et al., 2001; Devlin and Christopoulos, 2002). 2) AEA is a synthetic form of the endocannabinoid, anandamide which binds to the CB_1 receptors and, to a lesser extent CB_2 receptors. Anandamide is as potent as Δ^9-THC on CB_1 receptors (Grotenhermen, 2005; Pertwee and Ross, 2002). 3) 2-AG is a synthetic endocannabinoid that binds to both the CB_1

and CB_2 receptors with similar affinity, and is present at significantly higher concentrations in the brain (Stella et al., 1997; Pertwee, 2005).

Once dissolved, serial dilutions were made in the culture media from the 5mM stock solution of the drugs, and aliquots of 10nM, 50nM and 100nM solutions of Win, AEA and 2-AG, containing 4 x 10^{-6}%, 2 x 10^{-5}% and 4 x 10^{-5}% of DMSO were made. The effect of DMSO on the cells was not performed because, since the final concentration of DMSO was less than 0.01%, of which at this concentration, DMSO has been shown not to affect neuronal cells and glutamate levels in neuronal cell cultures (Ferraro et al, 2001; Otey et al., 2003; Fashola-Stone, 2005; Bottone et al., 2008). Glutamate has been shown to be the most common neurotransmitter in the brain and excessive glutamate release resulting in excessive Ca^{+2} entry into cells leads to excitotoxicity which initiates neuronal cell and brain ischaemic/hypoxic damage seen in stroke and cardiac arrest (Shigeri et al., 2004; Manev et al., 1989).

The drug concentrations were chosen by considering the concentrations used by other workers (Ferraro et al., 2001; Breivogel et al., 2004; Zhuang et al., 2005), and as such low dose (10nM), intermediate dose (50nM) and a higher dose (100nM) was selected to give a balanced view of the effect of the drugs from the low to a higher concentration. These solutions of the agonists were administered at 48hrs of culture to investigate the therapeutic effect of the agonists. Some cells were treated simultaneously at 0hr before they were exposed to the hypoxic incubator to assess the protective (pre-treatment) effect of the agonists against hypoxia. At each stage of the experimental period, the cells were observed under the microscope, using same field morphological assessment in which the culture plates were examined from the centre to the sides in a quadri-point analysis method (Ellingson et al., 2007; Sato and Momose-Sato, 2007), and this was repeated three times for each experiment. Micrographs of the cells were taken at the different time intervals (0hr to 120hrs), at a magnification of 200 times (x 200), using the Nikon Eclipse TS100 microscope and analysed using the IBM computer based Image Solution to investigate if changes have occurred between the normal and hypoxic cells in culture.

2.10.2 The effect of cannabinoid receptor agonists ± antagonists treatment on cellular proliferation in B50 cells in culture.

Introduction

Different bioactive agents have been shown to affect cellular proliferation and viability either negatively or positively. This study investigates the effect of treatment and pre-treatment with cannabinoid receptor agonists on cellular proliferation in B50 cells in culture using the Cell Titre 96 proliferation assay.

Materials

B50 neuronal cells, normal incubator, hypoxic incubator, Cell Titer 96® (Promega) assay kit, 12 well plates, and culture media, cannabinoid agonists (Win, AEA and 2-AG) at 10nM, 50nM and 100nM concentrations and cannabinoid antagonists (AM251 and AM630) at 10nM, 50nM and 100nM concentrations.

Method

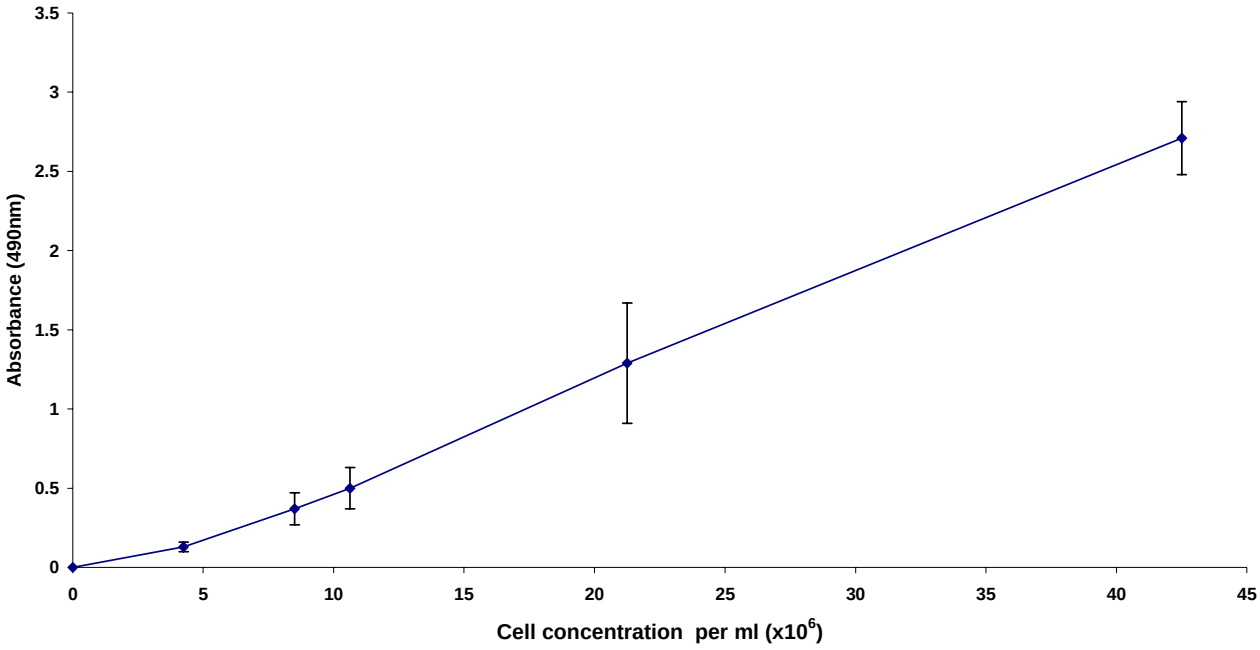
The B50 cells were raised in normal culture in 25cm^2 culture flask and then split into 12 well plates and were pre-treated immediately at 0hr with differing concentrations of the cannabinoid agonists as pre-treatment groups (simultaneous exposure groups) and cultured for 96hrs. The treatment groups were initially cultured for 48hrs and then treated with different concentrations of cannabinoid agonists in the presence or absence of cannabinoid antagonists for another 48hrs of culture. The cells were cultured for a total of 96hrs in culture. At the end of the time period in the incubators, the cells were harvested and assayed for cellular proliferation in B50 cell cultures, treated and pre-treated with cannabinoid agonists in the presence or absence of antagonists. The absorbance was read from the DYX MRX model of microplate reader and results calculated. Since it has been shown that cellular proliferation is directly proportional to the absorbance measured using the CellTiter One Solution (Xiong et al., 2007; Chett et al., 2007), the absorbance is used as an index of cellular proliferation. The results were calculated as follows.

a) Mean Absorbance = $\frac{\text{Sum of absorbances}}{\text{Number of assays (n= 6)}}$

b) Test Mean Absorbance= [Absorbance from the cells $_{(\text{Tests})}$ − Absorbance from the medium $_{(\text{Background})}$]

c) A standard curve was prepared using trypan blue cell counting method. A culture plate containing 42.5 x 10^6 cells/ml was used to prepared five dilutions (0, 2, 4, 5 and 10) x 10^6 cells/ml. Cells were transferred into microplate, treated with CellTiter Aqueous One Solution and absorbance (n=5) was measured at 490nm. The absorbance was plotted against the number of cells to give a standard curve as shown in Figure 2.4. The numbers of cells from the test groups were derived by plotting the absorbance of the test groups against the known cell numbers from the standard.

Fig.2.4. Standard curve of agonist treated B50 cell absorbance of the CellTiter One solution proliferation assay at 490nm as a function of cell concentration.



2.10.3 The effect of cannabinoid agonist ±antagonist on cellular viability using LDH release from neuronal B50 cells in culture.

Introduction

Kreutz et al. (2007), have shown the various effects of different cannabinoids in neuronal damage using activated microglial cells. Chen et al. (2005), have investigated the neuroprotective effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) using an *in vitro* model in which the AF5 CNS cell line was exposed to toxic levels of N-methyl-D-aspartate (NMDA) in which the cannabinoid antagonist SR141716A did not inhibit the neuroprotection induced by the Δ^9-THC, suggestive of a CB_1-independent protective mechanism. Louw et al. (2000), had shown a region specific protection by Δ^9-THC which implies that either the hippocampus undergoes suprathreshold ischaemic injury or that mechanisms of ischaemic injury vary in different brain regions. The aim of the study was to evaluate 1) the effect of cannabinoid agonist treatment or pre-treatment in the presence or absence of cannabinoid antagonists on neuronal viability using LDH assay. 2) the effect of different *in vitro* concentrations of the selective CB_1 receptor antagonist (AM251), that would antagonize the activities of a standard concentration of the cannabinoid agonist [Win 522,212-2 (10nM)], using LDH release from cultured neuronal B50 cells in treatment and pre-treatment groups.

Materials

LDH assay kit (Sigma 007); 1N hydrochloric acid; 12 well culture plates; 25cm^2 culture flasks and 96 well microplate; Centrifuge and MRX Microplate reader; B50 cells, normal and hypoxic incubators; Cannabinoid receptor agonists (Win, AEA and 2-AG), at 10nM, 50nM, 100nM; Cannabinoid antagonists (Tocris) [AM251 (No 117) and AM630 (No 1120)] at 10nM, 50nM and 100nM); Cannabinoid antagonist AM251 (1.0nM, 5.0nM, 10nM, 15nM, 20nM and 25nM).

Method

The B50 neuronal cells were resuscitated and cultured in 75cm^2 culture flasks in a normal incubator to confluency level of about 80-95% in 96hrs. The cells were split for sub-culturing in 12 well culture plates and the cannabinoid receptor agonists (Win, AEA and 2-AG in 10nM, 50nM and 100nM concentrations), were added to the

different wells at the same time as the cells were added to the wells (pre-treatment). The drugs were added to the cells simultaneously as the cells were exposed to the hypoxic condition at 0hr and cultured for a total of 96hrs. The cells in the treatment groups were cultured for 48hrs and then treated with the different concentrations of the agonists for another 48hrs. The cells pre-treated and treated with different cannabinoid agonists were cultured in different culture plates (Figure 2.5).

Some cells were treated or pre-treated with the agonist in the presence or absence of the antagonist at either 0hr for pre-treatment group or 48hrs for treatment group with the agonist (Win 10nM) and different concentrations of antagonist AM251 (1.0nM, 5.0nM, 10nM, 15nM, 20nM and 25nM). The cells were cultured for 96hrs in both treatment and pre-treatment groups. The cells were then harvested for LDH assay at 96hrs of culture and analysed. Tables 2.1 and 2.2, show the exposure time and the experimental conditions of the treatment and pre-treatment groups of cannabinoid agonists and antagonists. The LDH released was calculated as follows:

1) Total absorbance = Sum of (n) absorbances (n = 6)

2) Mean absorbance = $\frac{\text{Total absorbance}}{\text{Number of absorbances (n)}}$

3) Mean Corrected Absorbance= (Mean Absorbance–Mean Background Absorbance)

4) Percentage (%) Absorbance = $\frac{\text{Mean Test Absorbance}}{\text{Mean Control Absorbance}} \times 100$

Background Absorbance = absorbance from the normal culture medium only
Control Absorbance = Absorbance from the normal cells with medium
Corrected absorbance = Corrected Absorbance without any background
Test Absorbance = Absorbance from the experimental cells with the culture medium

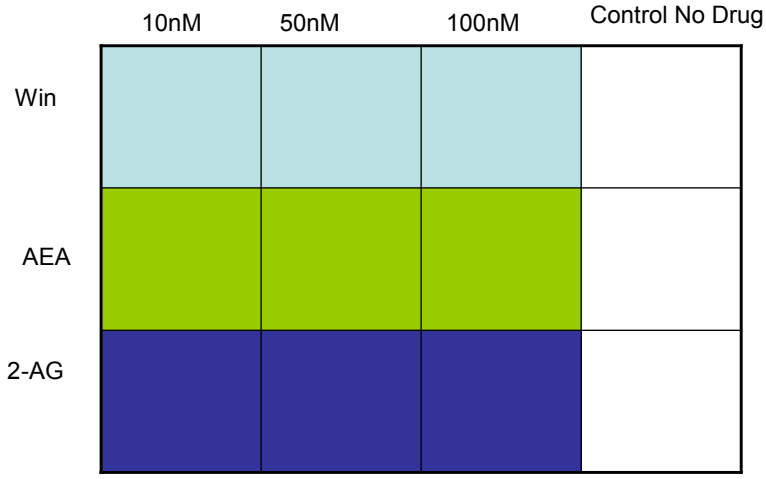


Fig.2.5. A 12-well culture plate showing the experimental conditions of each plate at the treatment or pre-treatment: Three cannabinoid agonists with different agonist concentrations

Table 2.1.Exposure time and experimental conditions of the treatment and pre-treatment groups of cannabinoid and opioid agonists in cultured B50 cells.

A)Drug treatment	Initial culture Time (hrs)	Treatment Time (hrs)	Total culture Time (hrs)
Win	48	48	96
AEA	48	48	96
2-AG	48	48	96
CONTROL	48	48	96
DAMGO	48	48	96
DSLET	48	48	96
ICI-199441	48	48	96
CONTROL	48	48	96

B)Drug pre-treatment	Initial culture time (hrs)	Pre-treatment Time (hrs)	Total culture Time (hrs)
Win	0	96	96
AEA	0	96	96
2-AG	0	96	96
CONTROL	0	96	96
DAMGO	0	96	96
DSLET	0	96	96
ICI-199441	0	96	96
CONTROL	0	96	96

Win: Win 55,212-2 mesylate, a potent non-selective CB_1/CB_2 receptor agonist with higher affinity for CB_1, AEA: Anandamide, a CB_1 receptor agonist; 2-AG: 2-arachidonylglycerol, a non-selective CB_1/CB_2 receptor agonist with same affinity. DAMGO:([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin, a Mu (μ) opioid receptor agonist
DSLET: [D-Ser^2, Leu^5, Thr^6]-enkephalin, a delta (δ) opioid receptor agonist
ICI-199441: ICI-199,441 hydrochloride, a kappa (κ) opioid receptor agonist
CONTROL: Experimental control

Table 2.2. Exposure time and experimental conditions in cannabinoid agonists/ antagonists treated and pre-treated groups of B50 cells in culture

A) Drug treatment Agonist/antagonist	Initial culture Time (hrs)	Treatment Time (hrs)	Total culture Time (hrs)
CONTROL/normal	48	48	96
Hypoxia no drug	48	48	96
10nM AM251/Win	48	48	96
50nM AM251/Win	48	48	96
100nM AM251/Win	48	48	96
10nM AM630/AEA	48	48	96
50nM AM630/AEA	48	48	96
100nM AM630/AEA	48	48	96
B)Drug Pre-treatment Agonist/antagonist	Initial culture time (hrs)	Pre-treatment Time (hrs)	Total culture Time (hrs)
CONTROL/normal	0	96	96
Hypoxia no drug	0	96	96
10nM AM251/Win	0	96	96
50nM AM251/Win	0	96	96
100nM M251/Win	0	96	96
10nM M630/AEA	0	96	96
50nM M630/AEA	0	96	96
100nM M630/AEA	0	96	96

AM251:1-(2,4-dichlorophenyl)-5-(4-iodophenyl]])-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide, a potent and selective CB_1 receptor antagonist. Win: Win 55,212-2 mesylate, a potent non-selective CB_1/CB_2 receptor agonist with higher affinity for CB_1 receptors, AM630:6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-y l] (4-methoxyphenyl)methanone, a non-selective CB_1/CB_2 antagonist with higher affinity for CB_2 receptors. AEA: Anandamide, a CB_1 receptor agonist;

2.11. The effect of opioid agonists on neuronal B50 cell morphology, proliferation and viability

2.11.1. The effect on morphology

Introduction

He et al. (2002), have shown that the ability of an agonist to promote endocytosis of μ opioid receptor (MOR) is not linearly related with agonist activity, indicating that MOR endocytosis is an independent functional property (Whistler et al., 1999; Whistler, et al., 2001). Delta opioid receptors have been shown to play an important role in delayed hypoxia preconditioning-induced neuroprotection against severe hypoxic injury (Zhang et al., 2006). The aim of this study was to evaluate the effect of opioid receptor agonists on the morphology of cultured neuronal B50 cells in hypoxia.

Materials

Three different highly potent synthetic opioid agonists were obtained from Tocris UK. These opioids agonist are DAMGO, (μ opioid), DSLET, (δ opioid) and ICI-199,441 hydrochloride, (κ opioid), normal and hypoxic incubators, culture media, 12 well culture plates, Nikon Eclipse TS100 Microscope using IBM Image solutions.

Method

The B50 cells were defrosted and resuscitated in the culture media for 24hrs. The three opioid receptor agonists [DAMGO (μ), DSLET (δ) and ICI (κ)], were dissolved separately in 100mM of 2%DMSO solution which formed the stock solution. Serial dilutions were made in the culture media and aliquoted into 10μM, 50μM and 100μM solutions of DAMGO, DSLET and ICI with DMSO concentrations of 0.0002%; 0.001% and 0.002% respectively. The effects of DMSO on the cells was not assessed since the concentration of DMSO in the drugs was less than 0.01% (Ferraro et al, 2001; Otey et al., 2003; Fashola-Stone, 2005; Bottone et al., 2008). The drugs were administered to the cells in cultures after 48 hours of initial culture to evaluate the therapeutic effects of the agonists on the cells for another 48 hours of culture, while some of the cells were simultaneously treated and exposed to hypoxic condition in the cultures at 0 hour to evaluate the protective effects of these agonists. All the cells

were cultured for a total of 96 hours in both normal and hypoxic conditions. The cells were examined as in 2.10.1 above.

2.11.2 The effect of opioid receptor agonists ± antagonists treatment on cellular proliferation in B50 cells in culture.

Introduction

Different bioactive agents have been shown to affect cellular proliferation and viability either negatively or positively. This study investigates the effect of treatment and pre-treatment with opioid receptor agonists on cellular proliferation in B50 cells in culture.

Materials

B50 neuronal cells, normal incubator, hypoxic incubator, Cell Titer 96® (Promega) assay kit, 12 well plates, and culture media, opioid agonists (DAMGO, DSLET and ICI-199,441) and opioid antagonists [CTAP (μ), ICI-174,864 (δ) and Nor (κ)] from Tocris at 10μM, 50μM and 100μM concentrations.

Method

The B50 cells were raised in normal culture in a $25cm^2$ culture flask and then split into 12 well plates and were pre-treated against hypoxia immediately at 0hr with differing concentrations of opioid agonists in the presence or absence of the antagonists as pre-treatment groups (simultaneous exposure groups) and were cultured for a total of 96hrs. The treatment groups were initially cultured for 48hrs and then treated with different concentrations of opioid agonists in the presence or absence of the antagonists for another 48hrs of culture to make up for a total of 96hrs of culture time. At the end of the period in the incubators, the cells were harvested and assayed for cellular proliferation in B50 cell cultures, treated and pre-treated with opioid agonists and antagonists, using CellTiter One Solution proliferation assay. The absorbance was read from the DYX MRX model of microplate reader. Since it has been shown that cellular proliferation is directly proportional to the absorbance measured using the CellTiter One Solution (Xiong et al., 2007; Chett et al., 2007), the

absorbance was used as an index of cellular proliferation. The results were calculated as follows.

a) Mean Absorbance = Sum of absorbances
Number of assays (n= 6)

b) Test Mean Absorbance= [absorbance from the cells $_{(Tests)}$-absorbance from the medium $_{(Background)}$]

c) A standard curve was prepared using trypan blue cell counting method. A culture plate containing 42.5 x 10^6 cells/ml was used to prepared five dilutions (0, 2, 4, 5 and 10) x 10^6 cells/ml. Cells were transferred into the microplate, treated with CellTiter Aqueous One Solution and absorbance (n=5) was measured spectrophotometrically at 490nm. The absorbance was plotted against the number of cells to give a standard curve as shown in Figure 2.4. The numbers of cells from the test groups were derived by plotting the absorbance of the test groups against the known cell numbers from the standard.

2.11.3 The effect of opioid agonist ± antagonist treatment on cellular viability using LDH release from B50 neuronal cells

Introduction

Opioid antagonists have been used to reverse the pharmacological actions of the opioid agonists. The present study examines the effects of the opioid receptor agonists in the presence or absence of the antagonists on cellular viability of B50 cells using LDH release.

Materials

Lactate dehydrogenase Assay kit (Sigma);12-well culture plates and culture media; normal and hypoxic incubators and centrifuge; 96 well Microplates and Dynex Microplate reader at 490nm; 1N hydrochloric acid (Sigma H9892); opioid agonists [DAMGO (μ), DSLET (δ), ICI-199,441 (κ)]; opioid antagonists [CTAP (μ), ICI-174,864 (δ) and Nor (κ)] from Tocris at 10μM, 50μM and 100μM concentrations.

Method

The B50 neuronal cells were raised in normal and hypoxic cultures for 48hrs, after which they were administered with the opioid receptor agonists at concentrations of 10μM, 50μM or 100μM for the treatment groups and cultured for another 48hrs for a total of 96hrs of culture, while the pre-treatment groups were treated at 0hr and then cultured for a total of 96hrs in culture. Some of the cells were treated with equivalent concentrations of the agonists and antagonists. The cells were processed for LDH release at the end of the culture period. The culture plates with the cells were removed from the incubator into the laminar flow hood. The plates were centrifuged at 250 x g for 4 minutes in order to pellet the cells. The supernatants were aliquoted into clean flat bottom 96 well microplates for the LDH procedure.

The LDH working solution was made by adding 25ml of deionized sterilized tissue culture water to the bottle of lyophilized cofactor. The lactate dehydrogenase assay mixture was prepared by mixing equal amounts (20ml) of LDH assay substrate, LDH assay cofactor and LDH assay dye solution. The LDH assay mixture was added to the aliquoted supernatant in the 96 well micro plates for assaying in a ratio of 2:1 (40μl:20μl). The plates were covered with aluminium foil to protect them from light and incubated for 30 minutes at room temperature. The reaction was stopped by the addition of one tenth volume (6μl) of 1N HCL to each well. The absorbance was measured spectrophotometrically at a wavelength of 490nm using DYX MRX model of micro plate reader. The exposure time and the experimental conditions of the treatment groups of opioid agonists are shown in Figure 2.6 and Tables 2.1 and 2.3. The LDH released from both control and experimental groups were calculated by subtracting the mean background absorbance of the wells from the mean primary absorbance of the test group and expressed as the percentage of the control group as shown below:

1) Total absorbance = Sum of (n) absorbances (n = 6)

2) $$\text{Mean absorbance} = \frac{\text{Total absorbance}}{\text{Number of absorbances (n)}}$$

3) Mean Corrected Absorbance= (Mean Absorbance–Mean Background Absorbance)

4) $$\text{Percentage (\%) Absorbance} = \frac{\text{Mean Test Absorbance}}{\text{Mean Control Absorbance}} \times 100$$

Background Absorbance = Absorbance from the normal culture medium only
Control Absorbance = Absorbance from the normal cells with medium
Corrected Absorbance = Corrected Absorbance without any background
Test Absorbance = Absorbance from the experimental cells with the culture medium.

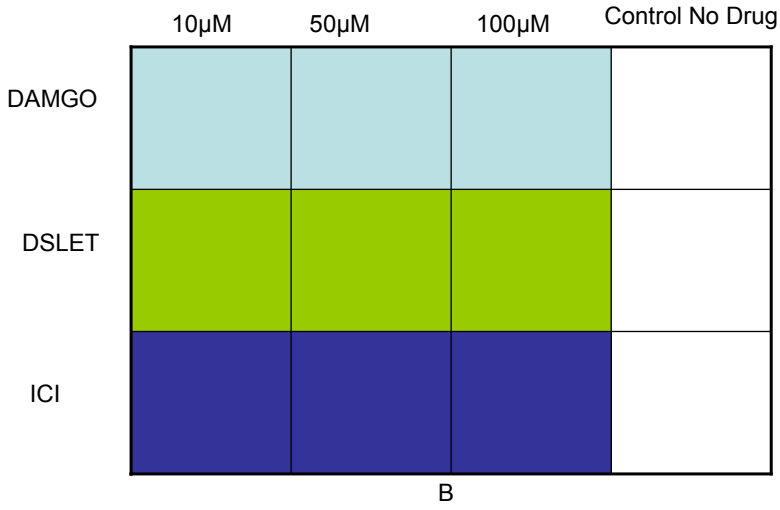


Fig.2.6. Diagram of 12-well culture plate showing the experimental conditions of each plate at the treatment and pre-treatment: Three opioid agonists in different treatment concentrations distinguished with three different colours.

Table 2.3. Exposure time and experimental conditions in opioid agonist/antagonist treated and pre-treated groups of B50 cells in culture.

A) Drug treatment Agonist/antagonist	Initial culture time (hrs)	Treatment time (hrs)	Total culture time (hrs)
Control/normal	48	48	96
10μM DAMGO/CATP	48	48	96
50μM DAMGO/CATP	48	48	96
100μM DAMGO/CATP	48	48	96
10μM DSLET/ICI-174,864	48	48	96
50μM DSLET/ICI-174,864	48	48	96
100μM DSLET/ICI-174,864	48	48	96
10μM ICI-199,441/NOR	48	48	96
50μM ICI-199,441/NOR	48	48	96
100μM ICI-199,441/NOR	48	48	96
B)Drug Pre-treatment Agonist/antagonist	Initial culture time (hrs)	Pre-treatment time (hrs)	Total culture time (hrs)
Hypoxia no drug	0	96	96
10μM DAMGO/CATP	0	96	96
50μM DAMGO/CATP	0	96	96
100μM DAMGO/CATP	0	96	96
10μM DSLET/ICI-174,864	0	96	96
50μM DSLET/ICI-174,864	0	96	96
100μM DSLET/ICI-174,864	0	96	96
10μM ICI-199,441/NOR	0	96	96
50μM ICI-199,441/NOR	0	96	96
100μM ICI-199,441/NOR	0	96	96

DAMGO: ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin, a Mu (μ) opioid receptor agonist CATP: D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; a selective μ-opioid receptor antagonist, DSLET: [D-Ser^2, Leu^5, Thr^6]-enkephalin, a δ-opioid receptor agonist, ICI-174,864: ([allyl]2-Tyr-alpha-amino- isobutyric acid-Aib-Phe-Leu-OH), a selective δ-opioid receptor antagonist. ICI-199,441: ICI-199,441 hydrochloride, κ-opioid receptor agonist. NOR: Norbinaltorphimine, a potent κ-opioid receptor antagonist.

2.12 The effect of DbcAMP on LDH release from B50 cells treated with cannabinoid and opioid agonists ± antagonists.

2.12.1 Introduction

Some agents have been shown to stimulate cell differentiation while some are known to cause cellular death and degeneration (Boghaert et al., 1991). Some cell types exhibit increased numbers of long cellular processes known as morphological differentiation following treatment of monolayer cultures with 1mM dibutyryl cyclic adenosine monophosphate (DbcAMP) (Hu et al., 2007; Oda et al., 2007). It has been shown that DbcAMP induces neuronal differentiation by inhibition of cellular proliferation, followed by neurite outgrowth (Kim et al., 2002). It has also been shown that mitogen activated protein kinase (MAPK) signalling is required for neurite outgrowth in neuronal cells and hence dbcAMP-induced neurite outgrowth in cells, may be by amplifying both the MAPK-dependent and -independent DbcAMP signalling pathways (Li et al., 2003). The aim of this study was to investigate the effect of DbcAMP on LDH release from B50 cells treated with cannabinoid and opioid agonists in the presence or absence of antagonists in hypoxic cultures.

2.12.2 Materials

Cannabinoid agonists at 10nM, 50nM and 100nM concentrations; opioid agonists at 10μM, 50μM and 100μM concentrations; cannabinoid antagonists (AM251 and AM630) at 10nM, 50nM and 100nM; opioid antagonists (CTAP for μ, ICI-174,864 for δ, Nor for κ) at 10μM 50μM and 100μM; B50 cells, 12 well plates and 25cm^2 culture flasks; LDH kit, normal and hypoxic incubators.

2.12.3 Method

The B50 cells were raised in normal culture and split at full confluency. The split cells were subcultured and treated with 1mM DbcAMP at 0hr. The different group of cells were treated for 48 hrs after the initial culture of 48hrs, or pre-treated at 0hr for 96hrs with the different concentrations of cannabinoid agonists and antagonists, or opioid agonists and antagonists. At the end of 96 hrs of culture, the cells were harvested and the effect of DbcAMP on the treatment and pre-treatment with cannabinoid agonists ± antagonists and opioid agonists ± antagonists were assessed by LDH release using the LDH assay. The results were calculated using the LDH formulae shown in Section 2.11.3. The exposure time and the experimental conditions are shown in Table 2.4.

Table 2.4. Exposure time and experimental conditions on the B50 cells treated with DcAMP/cannabinoid and opioid agonists ± antagonists in culture.

A) Cannabinoid agonist treatment	Initial culture time (hrs)	Treatment/pre-treatment time (hrs)	Total culture time (hrs)
Control/normal	48	48	96
Hypoxia no drug	48	48	96
DbcAMP	48	48	96
DbcAMP/10nM Win	48	48	96
DbcAMP/50nM Win	48	48	96
DbcAMP/100nM Win	48	48	96
B) Opioid agonist treatment	Initial culture time (hrs)	Treatment time (hrs)	Total culture time (hrs)
DbcAMP/10µM DAMGO	48	48	96
DcAMP/100µm DSLET	48	48	96
DbcAMP/10µM ICI-199,441	48	48	96
DbcAMP/100µM ICI-199,441	48	48	96
C) Opioid agonist pre- treatment	Initial culture time (hrs)	Pre-treatment time (hrs)	Total culture time (hrs)
DbcAMP/10µM DAMGO	0	96	96
DbcAMP/100µM DAMGO	0	96	96
DcAMP/10µM DSLET	0	96	96
DbcAMP/100µm DSLET	0	96	96
DbcAMP/10µM ICI-199,441	0	96	96
DbcAMP/100µMICI-199,441	0	96	96
D) Cannabinoid/ Antagonist treatment	Initial culture time (hrs)	Treatment time (hrs)	Total culture time (hrs)
DbcAMP/10nMAM251/ Win	48	48	96
DbcAMP/50nMAM251/ Win	48	48	96
DbcAMP/100nMAM251/ Win	48	48	96

DbcAMP: dibutyryl cyclic adenosine monophosphate; Win: Win 55,212-2 mesylate, a potent non-selective CB_1/CB_2 receptor agonist with higher affinity for CB_1 receptors, DAMGO:([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin, a µ opioid receptor agonist
DSLET: [D-Ser^2, Leu^5, Thr^6]-enkephalin, a delta (δ) opioid receptor agonist
ICI-199441: ICI-199,441 hydrochloride, a kappa (κ) opioid receptor agonist
AM251:1-(2,4-dichlorophenyl)-5-(4-iodophenyl]])-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide, a potent and selective CB_1 receptor antagonist

2.13 The effect of hypoxia on cAMP levels in cultured B50 cells: cAMP enzyme immunoassay (ELISA) method.

2.13.1 Introduction

Adenosine 3'5'-cyclic monophosphate (cAMP) form intermediate signals in cellular processes, making them popular targets for research and drug discovery processes. There has been considerable interest in the measurement of intracellular cAMP in tissues and cell cultures and this may help to provide an understanding of the physiology and pathology of many disease conditions (Tremblay et al., 1988; Sevetson et al., 1993; Morton, 2004; Sigma, 2006). The aim of this study was to evaluate the effect of hypoxia on the level of intracellular cAMP content in normal, hypoxic and treated neuronal B50 cells in culture using the direct cAMP enzyme-linked immunosorbent assay (ELISA) method. The direct cAMP ELISA have been validated and shown to be a reliable method for the quantification of intracellular cAMP in cultured cells (Kim et al., 2006; Qiu et al., 2002; Cai et al., 2001; Andersen et al., 2001).

2.13.2 Materials

Cultured B50 cells in normal and experimental groups, Sigma direct cAMP enzyme immunosorbent assay kit (CA-200) containing the followings; Goat anti-Rabbit IgG microtiter plates, cAMP direct conjugate, cAMP direct Antibody, 0.1M HCL, Neutralizing reagent, Wash buffer concentrate, cAMP standard, p-NPP substrate, Stop solution, Triethylamine, acetic anhydride and Micro plate sealer, deionized water, Pipettes, Micro plate shaker, adsorbent paper for blotting and Micro plate Reader at 410 nm.

2.13.3. Method

The B50 neuronal cells were cultured in 12-well culture plates in both normal and hypoxic incubators. The cells were treated with cannabinoid and opioid agonists at 10nM, 50nM and 100nM concentrations for cannabinoid and 10μM, 50μM and 100μM concentrations for opioid agonists. At the end of 96hrs of culture, the culture media were decanted and the cells were treated with 0.1M HCL and incubated for about 15 minutes. The cells were visually inspected to verify complete cell lysis. The cells in the wells were centrifuged at 600 x g at room temperature for 5 minutes. The supernatants were then collected and used directly for cAMP assay.

Cyclic AMP standard solutions were prepared in 5 glass tubes (1 to 5). 900µl of 0.1M HCl was added to tube 1, while to tubes 2-5 were 750µl of the 0.1M HCl added. Then 100µl of the 2,000pM/ml Standard was added to tube 1 and mixed thoroughly. 250µl of tube 1 was added to tube 2 and mixed. This was continued for tubes 3 through tube 5 as in Table 2.5. Wash buffer was prepared by diluting 10ml of the wash buffer concentrate with 90ml of deionized water.

Then 50µl of the neutralizing reagent was pipetted into each well except the Total activity (TA) and Blank wells. 100µl of the 0.1M HCl was added into Non-specific binding (NSB) and Blank 0 Standard (Bo) wells and 100µl of the standard tubes 1 through 5 was added into standard wells. Then 100ul of the cell supernatant samples were added into sample wells and 50ul of 0.1M HCl was added to NSB wells. Also 50µl of cAMP conjugate was added into each of the wells except the TA and Blank wells. Then 50µl of cAMP antibody was added into each well except the Blank, TA and NSB wells. The well arrangements are shown in Table 2.6.

The plate was incubated at room temperature for 2 hours on a plate shaker at 500rpm with the plates covered with the plate sealer. At the end of the incubation period, the contents were emptied and washed 3 times by adding 200µl of the wash solution to every well. At the end of the washing, the wells were emptied completely to remove all wash buffer. Then 5µl of the cAMP conjugate was added to the TA wells and 200µl of the p-NPP substrate solution was added to every well and incubated at room temperature for one hour without shaking. At the end of the incubation period, 50µl of the stop solution was added to every well and the optical density of the plate was read at 410nm. Each assay was performed in triplicate and repeated twice (n=6). Calculation of the cAMP concentration in the cell supernatant samples was done as follows:

- Average Bound OD = $\frac{\text{Sum of the optical density of the sample wells}}{\text{Number of wells (n=6)}}$
- Average NSB OD = $\frac{\text{Sum of the optical density of non specific binding wells (Background)}}{\text{Number of wells (n=6)}}$
- Net Optical Density (Net OD) = (Average Bound OD) – (Average NSB OD)
- Net Bo OD = (Average Bo - Average NSB OD)

- Percent Bound = $\frac{\text{Net Optical Density (OD)}}{\text{Net Bo OD}}$ X 100

- Using graph paper plot percent bound from the maximum binding wells (B/Bo) versus the known cAMP concentrations from the standard to generate a standard curve as shown in Figure 2.7 from where the cAMP concentration from the unknown experimental groups were derived.

Where OD = Optical density
Net OD = Mean optical density
NSB OD = Non-specific binding optical density
Net Bo OD = Maximum binding wells

Table 2.5. Dilution table for making cAMP Standards 1-5:

Standards	0.1MHCL Vol.(µL)	cAMP Standard Vol. Added(µL)	cAMP(Conc.) (pmol/mL)
1	900	100, Stock	200
2	750	250, Std. 1	50
3	750	250, Std 2	12.5
4	750	250, Std 3	3.12
5	750	250, Std 4	0.78

Fig.2.7. Standard curve of B50 cell optical density (410nm) for determination of cAMP concentration

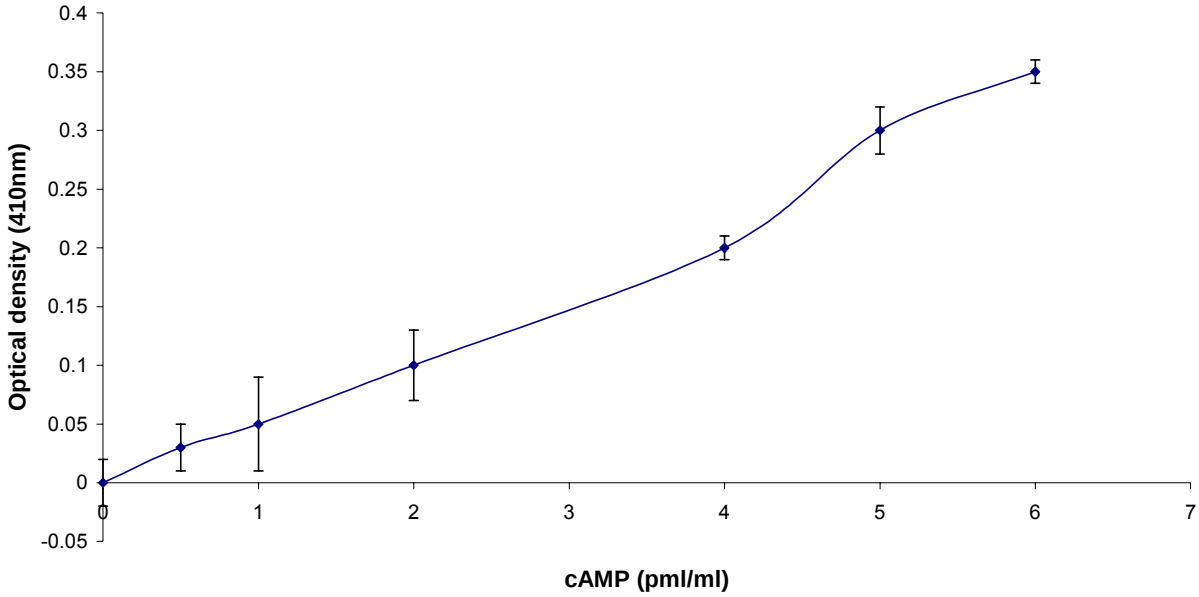


Table 2.6: cAMP Assay Protocol Flow Chart

	Blank	TA	NSB	Zero Std (Bo)	Stds	Samples
Well Type and reagent added	A1,B1	C1,D1	E1,F1	G1,H1	A2-B3	C3-H12
Neutralizing reagent	-------	-------	50µL	50µL	50µL	50µL
0.1M HCL	-------	-------	150µL	100µL	-------	-------
Standard & Sample	---------	-------	-------	--------	100µL	100µL
Conjugate	---------	------	50µL	50µL	50µL	50µL
Antibody	---------	-------	--------	50µL	50µL	50µL
Incubate for 2hrs at room temperature with shaking	_____	_____	_____	_____	_____	_____
Wash with 3x200µL of washing solution	_____	_____	_____	_____	_____	_____
Conjugate	-------	50µL	------	-------	-------	-------
Substrate	200µL	200µL	200µL	200µL	200µL	200µL
Incubate for 1hr at room temperature without shaking	_____	_____	_____	_____	_____	_____
Stop Solution	50µL	50µL	50µL	50µL	50µL	50µL

TA Total activity
NSB Non specific binding
Bo Maximum binding
Std Standard

2.14 The effect of hypoxia on extracellular signal regulated protein kinases (ERK) in cultured B50 neuronal cells: ERK1/2 enzyme immunoassay (ELISA) method

2.14.1 Introduction

Increased extracellular signal-regulated protein kinases (ERK1&2) phosphorylation has been noted in vulnerable penumbra following acute ischaemic stroke in human, in addition to other chronic neurodegenerative diseases (Grewal, et al., 2000: Liebmann and Bohmer, 2000; Wang et al., 2004). The aim of this study was to investigate the effect of hypoxia on phospho-ERK1/2 in cultured neuronal B50 cells.

2.14.2. Materials

Cultured normal, hypoxic and treated neuronal B50 cells; Sigma ERK1&2 (EK0100) ELISA kit containing: ERK1&2 Standard, Standard diluent buffer, monoclonal Anti-ERK1&2-coated well plates, Anti-ERK1&2, Anti Rabbit IgG-HRP, HRP diluent, Wash buffer concentrate, Stabilized chromogen, Tetramethylbenzidine (TMB), Stop solution, Plate covers and Adhesive strips; MRX Micro plate reader at 450nm; Pipettes, deionized water, Plate washer, Glass tubes, Absorbent paper towels for blotting plate, Vortex mixer; Sigma RIPA cell lysis buffer (N080-1284); Phenylmethlsulfonyl fluoride (PMSF)(Sigma P7626); Protease Inhibitor cocktail (PIC) (Sigma P8340); Sodium orthovanadate (Sigma S6508); Sodium pyrophosphate (Sigma S6422), Phosphate buffered saline(PBS).

2.14.3 Method

Protein Extraction from B50 Cells

Materials

The cell extraction buffer was made by the addition of 20mM sodium pyrophosphate, 2mM sodium orthovanadate, while 1mM Phenylmethlsulfonyl fluoride (PMSF) and 500µl of protease inhibitor cocktail (PIC), was added to every 10ml of the cell extraction buffer, prior to use. Rubber scrapers were used to remove the cells from the culture plate. The cell extraction buffer was prepared on ice.

Method of cell extraction

The B50 ells are adherent and were removed from the plate by scraping the culture plate with the rubber scrapper and washed twice with cold phosphate buffered saline (PBS) and centrifuged at 2500 x g for 5 minutes. The supernatant was removed leaving the cell pellets. The cell pellets were lysed using 500µl of the made-up cell extraction buffer for 30mins on ice with vortexing every 10mins intervals. The extracts were transferred into micro centrifuge tubes and centrifuged at 13,000rpm for 10 minutes. The lysates were aliquoted into clean micro centrifuge tubes.

Pre-treatment of cell extracts before assay.

The cell extracts were heated in boiling water for 5 minutes, upon cooling, the extracts were centrifuged and equal aliquots were used for the ERK1/2 ELISA Assay.

Reagent Preparation for ERK1/2 ELISA

Standard Dilution

The ERK1/2 standard was reconstituted by adding 0.88ml of standard diluent buffer into one vial of the standard. The solution was mixed gently and allowed to stay for 10 minutes to ensure complete reconstitution. This was labelled as 100Units/mL ERK1/2. The serial dilutions of the standard were made as shown in Table 2.7

Anti-ERK1/2 antibody

Monoclonal antibody specific for ERK1/2 was supplied as monoclonal Anti-ERK1/2-coated well plates in the assay kit (Sigma E2655), while the detection antibody specific for the phospho-ERK1/2 was supplied as phospho-Anti-ERK1/2 (Sigma E4155) ready to use.

Anti-Rabbit IgG-HRP

The Anti-Rabbit IgG-HRP concentrate was allowed to equilibrate to room temperature. 10µl IgG-HRP concentrate was mixed with 1ml of HRP diluent to make-up 1:100 dilution which is sufficient for 8 well strips. This was labelled as Anti-Rabbit IgG-HRP working solution and the unused concentrate returned to the refrigerator.

Wash buffer

The wash buffer concentrate was allowed to equilibrate to room temperature and one volume of the wash buffer concentrate was mixed with 24 volumes of deionized water. This was labelled as the working wash buffer and both the concentrate and the working wash buffer was then put in the refrigerator until when needed.

2.14.4 Method for phospho-ERK 1&2 assay in B50 cells

Well Arrangement

The number of wells for the assay was determined by adding together 2 Blank/Zero wells, 2 Chromogen Blank wells, 14 Standard dilution wells and 2 wells for each of the samples to be assayed. Appropriate number of multi well strips removed, while the remaining was resealed and returned to the refrigerator. The layout of the wells is as shown in Table 2.8.

Assay incubation

100µl Standard diluent was added to Zero wells and 100µl Standards, Samples or Controls were added to appropriate wells as shown in Table 2.8. The plate was tapped gently to mix and was covered with the plate cover and incubated for 2hrs at room temperature as the first incubation step. At the end of the incubation, the wells were washed 4 times with the working wash buffer. After the final wash, the plates were blotted dry to remove any remaining wash buffer. 100µl of Anti-ERK1/2 detection antibody (Sigma E 4155), which was supplied ready to use, was added to all the wells except Chromogen Blank wells and the plate was tapped gently to mix, covered with the plate cover and incubated for 1hr at room temperature as the second incubation. The wells were washed 4 times and blotted dry to remove any remaining wash buffer. Then 100µl Anti-Rabbit IgG-HRP working solution in a dilution of 1:100, was added to each well except Chromogen Blank wells. The plate was covered and incubated for 30 minutes at room temperature as a third incubation. The wells were washed 4 times and blotted dry. Then 100µl of Stabilized Chromogen was added to all the wells and the wells turned blue. The plate was left open and incubated for 30 minutes at room temperature in the dark as the substrate incubation. Finally 100µl Stop solution was added to all the wells to stop the reaction. The plate was tapped gently to mix and the solution turned yellow at this point. The assay was done in triplicate and repeated

twice (n = 6). The absorbance of the plate was read at 450nm on MRX Micro plate reader.

Calculation of ERK concentration

The concentrations of ERK in the different groups of the B50 cells were calculated using the formulae below;

1) Average Optical density = Sum of the optical densities

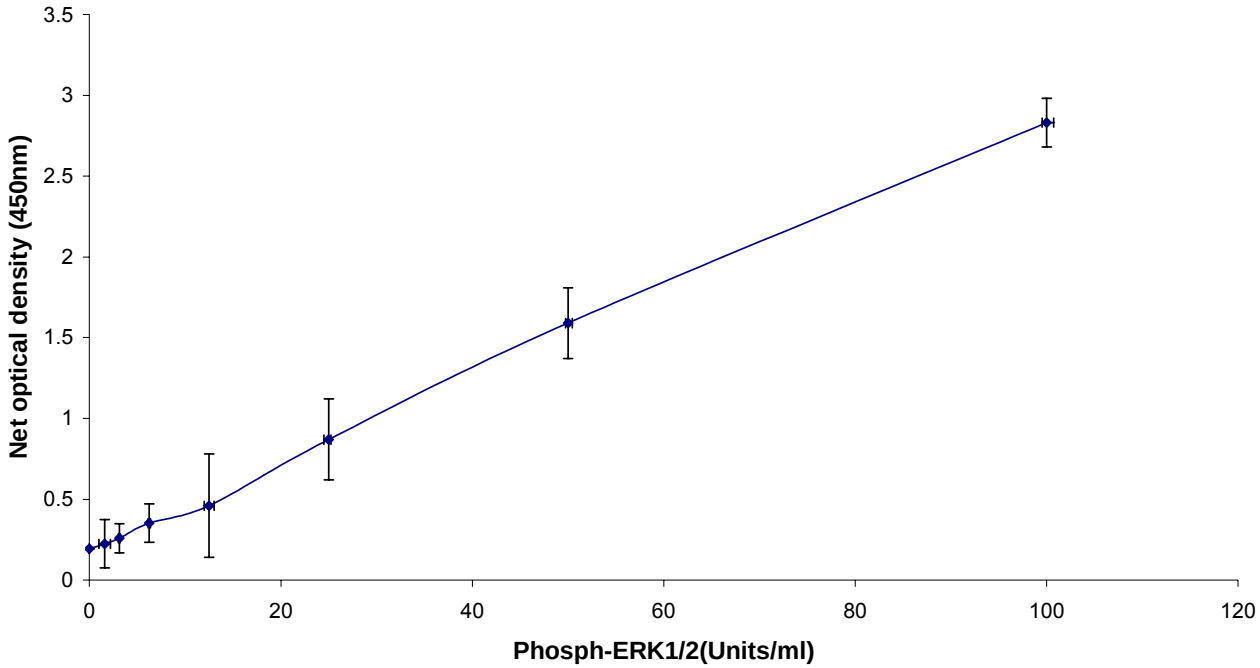
Number of wells (n = 6)

2) Net OD = (Average Bound OD –Average Chromogen Blank OD)

3) Net OD (nm) of the standard dilutions was plotted against the standard concentration (Units/mL) of phospho-ERK1& 2 and the best curve was drawn to give a standard curve (Figure 2.8) through which the concentrations of the phospho-ERK1/2 in the test groups were derived.

OD= Optical density
Net OD= Net optical density

Fig.2.8. Quantitative determination of phospho-ERK in standards



2.15. Statistical Analysis

The different parameters measured from the normal, hypoxic, treated and pre-treated experimental groups of cultured B50 neuronal cells were compared using mean and standard deviation (SD). The parameters were assayed in triplicate and repeated twice (n = 6) and the results presented as the mean ± SD. The Students' t-test was used for testing the level of significance between two groups and a P-value less than 0.05 was considered to be significant using Microsoft Excel® package. For multiple treatment data, One-Way Analysis of Variance (ANOVA) was used followed by Multiple Range Test post hoc subgroup testing to find the least significant difference (LSD) between the groups.

Table 2.7. Dilution for making ERK1 & 2 standards

Tube No	Standard Diluent Buffer	Standard from tube Number	Final(ERK1&2) Units/mL
1	0 .88mL	_____	100U/mL
2	0.25mL	0.25mL (1)	50
3	0.25mL	0.25mL (2)	25
4	0.25mL	0.25mL (3)	12.5
5	0.25mL	0.25mL (4)	6.25
6	0.25mL	0.25mL (5)	3.12
7	0.25mL	0.25mL (6)	1.6
8	0.25mL	_____	0

Table 2.8 Phospho-ERK 1 & 2 assay protocol flow chart

	Blank A1, B1	Chromogen Blank C1, D1	Standards G1 to B3	Samples C3 to H12
Std Diluent	100µl	---------	--------	-------
Stds/Samples	--------	---------	100µl	100µl
Incubate for 2 hrs at room temperature	_____	_____	_____	_____
Wash 4 times with washing solution	_____	_____	_____	_____
Anti-ERK1&2	100µl	---------	100µl	100µl
Incubate for 1hr at room temperature	_____	_____	_____	_____
Wash 4 times with washing solution	_____	_____	_____	_____
Anti-RabIgG HRP	100µl	---------	100µl	100µl
Incubate for 30 mins at room temperature	_____	_____	_____	_____
Wash for 5 times with washing solution.	_____	_____	_____	_____
Chromogen	100µl	100µl	100µl	100µl
Incubate for 30 mins at room temperature.	_____	_____	_____	_____
Stop solution	_____	_____	_____	_____

Std = Standard
Stds = Stangards
Anti = Antibody
RabIgG HRP = Rabbit immunoglobulin G-Horse radish peroxidise
Hrs = Hours
Mins = Minutes
A1-H12 = Plate number
Stop solution = Ready to use solution that stop the reaction in the plate

CHAPTER THREE

3. Results

3.1. Cell characteristics

The effects of hypoxia on neuronal cell signalling and the consequent neuroprotectant effects of cannabinoid and opioid agonists against hypoxia were investigated using cortical B50 neuronal cells that were cultured in both normal and hypoxic incubators. Cannabinoid and opioid receptor agonists were administered during the treatment and pre-treatment against hypoxia and the effects and cellular changes were studied using different parameters.

3.1.1 B50 cell culture

The B50 neuronal cells were initially cultured in normal incubator in normoxic conditions (21% O_2; 5% CO_2) in order to raise normal B50 cells for continuous sub-culturing during the course of the research. The initial B50 cell culture was used to raise 100 vials (1ml ampoules) of normal B50 cells which were frozen and stored at -70^{o}C, from which subsequent cultures were raised for various experimental studies conducted in both normal and hypoxic incubators. The B50 cells cultured in the normal incubator were used as the control cells, while those cultured in the hypoxic (5% O_2; 5% CO_2) incubator were used as B50 cells in hypoxia which underwent therapeutic and protective treatments under different experimental conditions.

3.1.2. Morphological studies

Morphological changes were observed in B50 cells cultured under hypoxia when compared with those cultured in normal incubator. The B50 cells in hypoxia showed clustered groups of neuronal B50 cells, evidence of degenerating, dying cells and already degenerated and dead neuronal B50 cells. The normal B50 neuronal cells cultured under normal incubator showed normal neuronal morphology as shown in Plates 1 and 2, when compared to B50 cells in hypoxia as shown in Plates 6, 7 and 8.

3.1.3. Pattern formation

The results from the pattern and pattern formation experiments showed the B50 neuronal cells in the normal incubator with normal neuronal pattern and tract formation resembling those nerve fibres in normal neuronal cellular development in the body, when compared to the disoriented pattern found in B50 cells cultured under hypoxia. The normal neuronal pattern and pattern formation is shown in Plates 3,4 and 5, when compared to the abnormal pattern found in hypoxic cells as shown from Plates 6, 7 and 8.

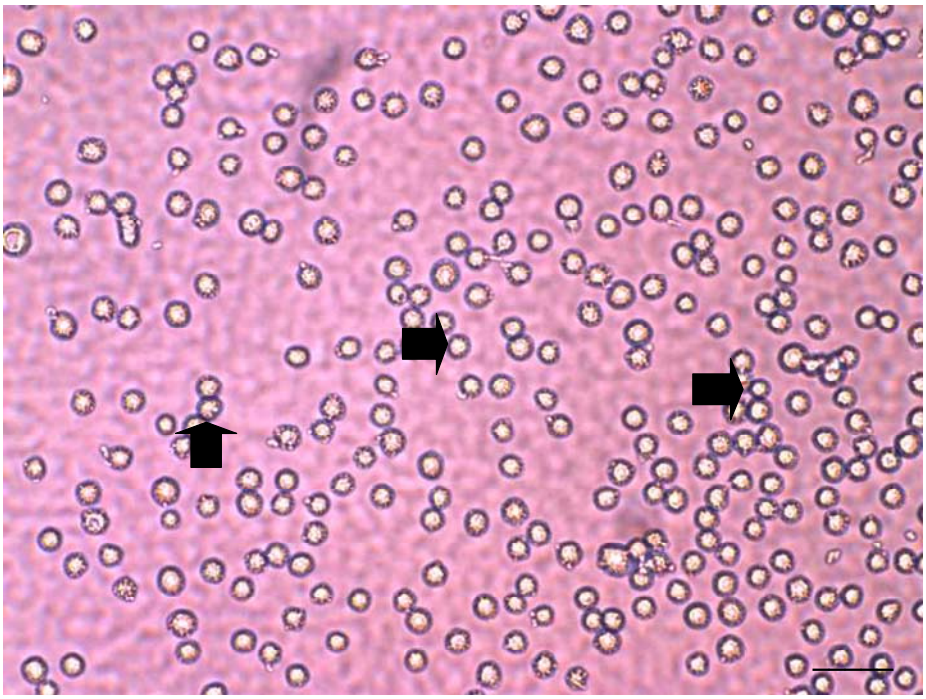


Plate 1:Representative of B50 cells at 0hrs with normal B50 cells (arrow) at the point of starting the culture at 21% O_2 and 5% CO_2. B50 cells were observed in three different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mmx40 magnification.

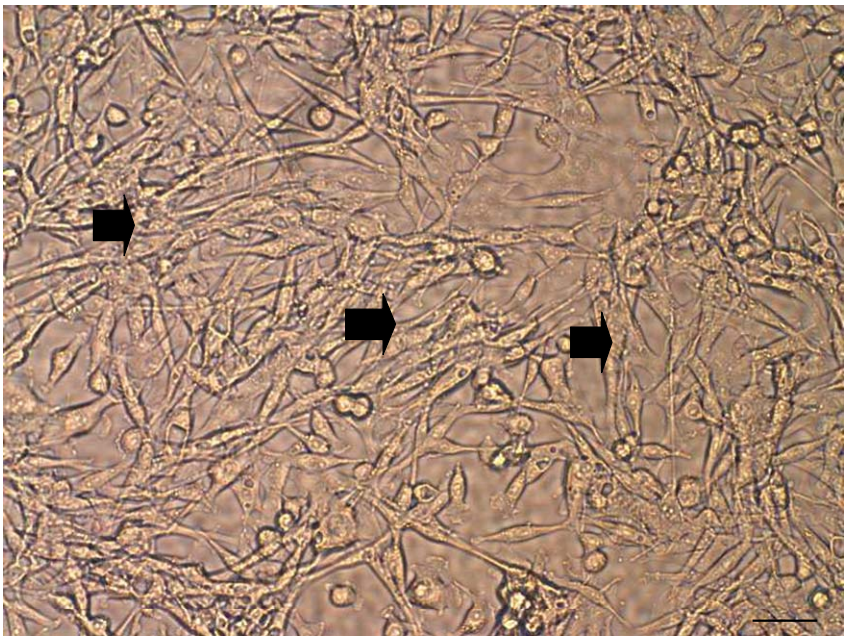


Plate 2:Representative of B50 cells at 48hrs of normal culture (21%O_2 and 5% CO_2) with B50 cells (arrow). B50 cells were observed in three different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with IBM Image Solutions®. Scale bar =5mmx40 magnification.

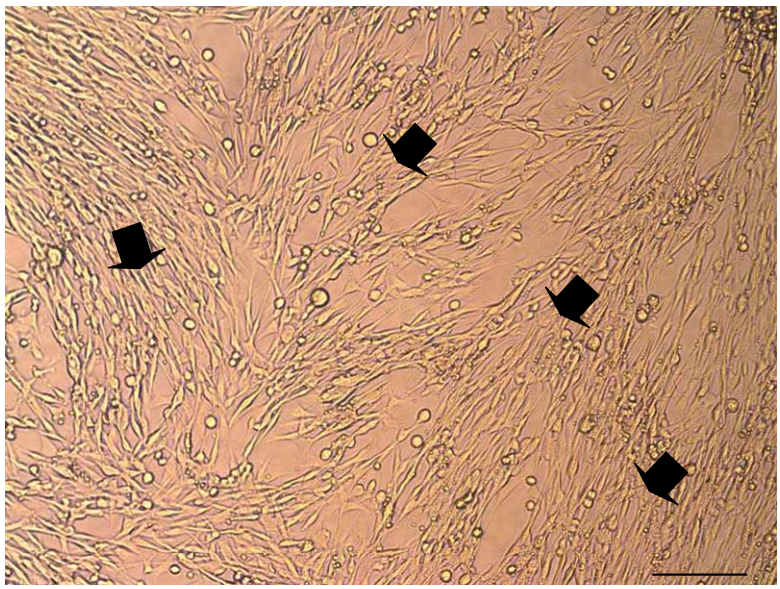


Plate 3:Representative of normal B50 cells at 48hrs of culture (21%O_2 and 5% CO_2) with normal pattern (arrow). Pattern of groups of nerves in a particular direction were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx20 magnification.

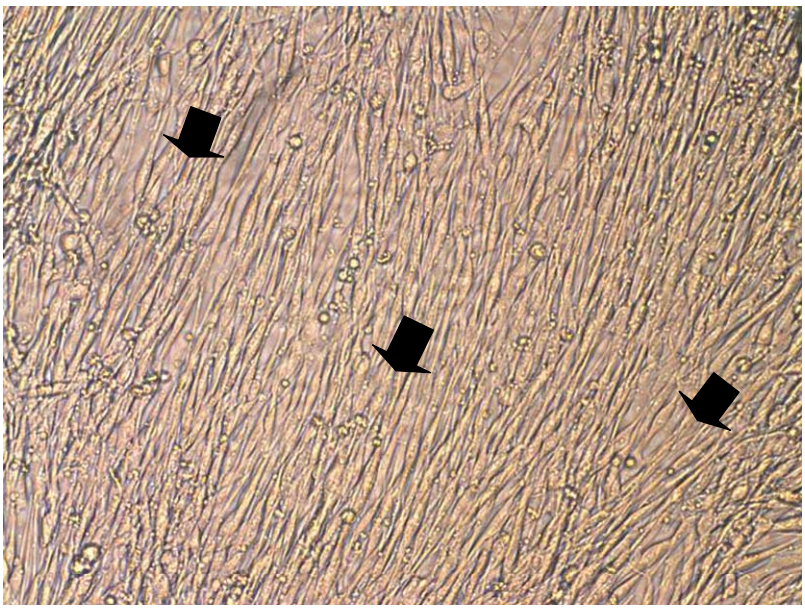


Plate 4:Representative of normal B50 cells at 96hrs of culture (21%O2 and 5% CO2) with normal pattern (arrow). Pattern of groups of nerves in a particular direction were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

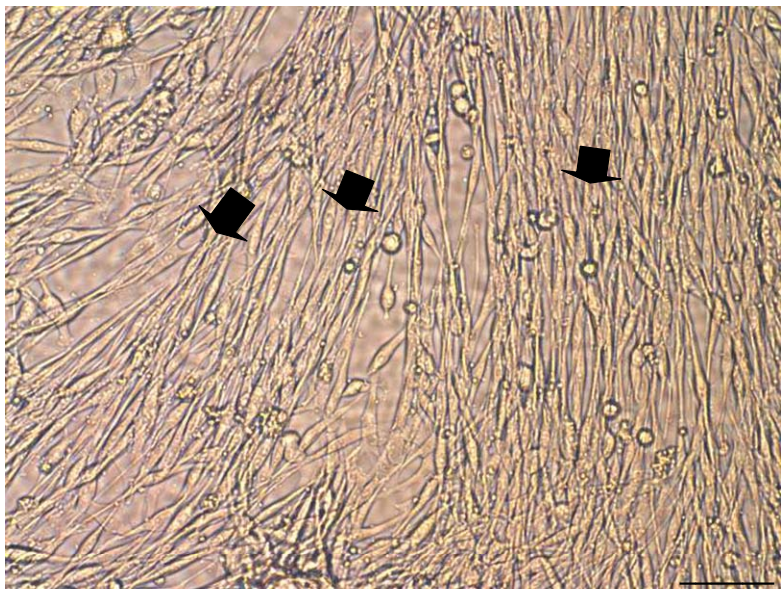


Plate 5:Representative of normal B50 cells at 96hrs of culture (21%O_2 and 5% CO_2) with normal pattern (arrow). Pattern of groups of nerves in a particular direction were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification

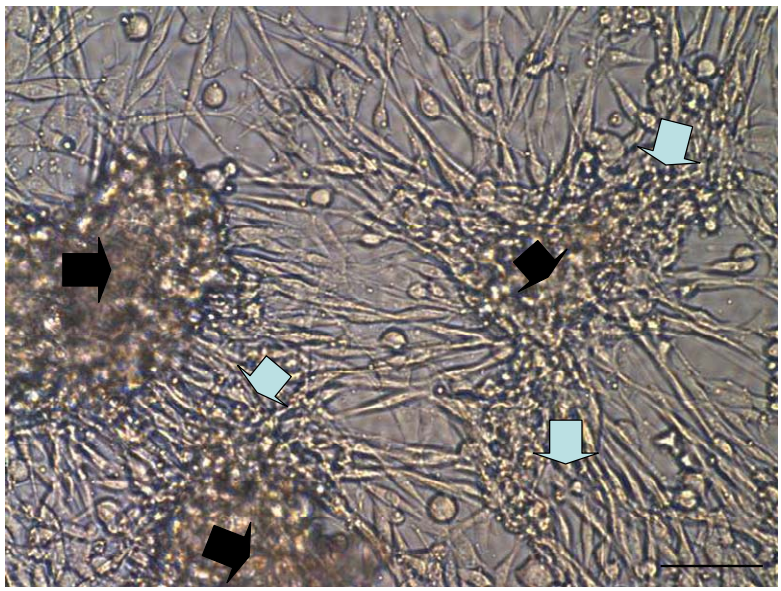


Plate 6:Representative of hypoxic B50 cells at 96hrs of culture (5%O_2 and 5% CO_2), with groups of degenerating cells (black arrow) and altered pattern of cell arrangement (blue arrow). Groups of degenerating and altered nerve arrangement were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

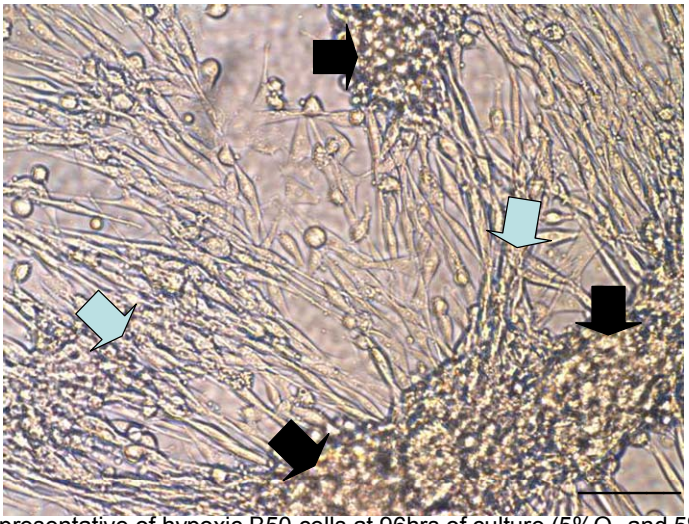


Plate 7:Representative of hypoxic B50 cells at 96hrs of culture (5%O_2 and 5% CO_2), with groups of degenerating cells (black arrow) and altered pattern of cell arrangement (blue arrow). Groups of degenerating and altered nerve arrangement were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

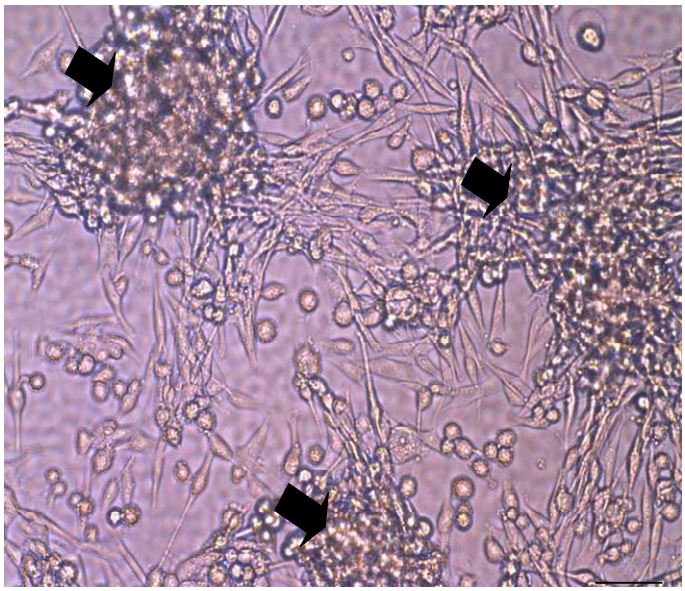


Plate 8:Representative of hypoxic B50 cells at 48hrs of culture (5%O_2 and 5% CO_2) without drug administration, showing groups of degenerating cells (arrow). Groups of degenerating cells were observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification

3.1.4 Cell count and viability

The time course effect of hypoxia on total cell count and viability showed that at 0 hour, the mean total cell count in B50 neuronal cell culture was 2.67±1.64x10^6cells/ ml, the total viable cells was 2.50±1.40x10^6cells/ml, while the percentage viability was 93.64±2.79% and the range of percentage viability was 90-97%. There was no difference in the total cell count and total viable cells at 0 hour of culture, between the normoxic and hypoxic cells since the cells were split from the normal cells which serve as 0 hour at the beginning of the culturing experiment. The 0 hour serves as the base line for the culture of both normoxic and hypoxic cells.

At 24 hours, the normal cultured B50 cells had a total cell count of 3.51±0.87x10^6 cells/ml, total viable cells 3.05±0.83x10^6 cells/ml, with 87.14±0.72 as percentage viability and range of 86-88% while B50 cells cultured under hypoxia had 2.80±0.36x10^6cells/ml; 1.95±0.24x10^6cells/ml; 70.91±5.41% and 63-76% as total cell count, total viable cells, percentage viability and range of percentage viability, respectively. The difference in the total cell count and total viable cells between the normal and hypoxic cells was not statistically significant.

At 48 hours of culture, the normal cultured B50 cells had 6.50±0.84 x10^6cells/ml; 6.01±0.85 x 10^6cells/ml; 92.25 ±1.17%; 90-93%, as total cell count, total viable cells, percentage viability and range of percentage viability respectively, while the hypoxic cells had 5.34±1.45 x 10^6cells/ml, 3.89±1.22 x10^6cells/ml, 72.01±3.39% and 67-76% as total cells count, total viable cells, percentage viability and range of percentage viability, respectively. The difference in the total cell count and total viable cells between the normal and hypoxic cells was not statistically significant.

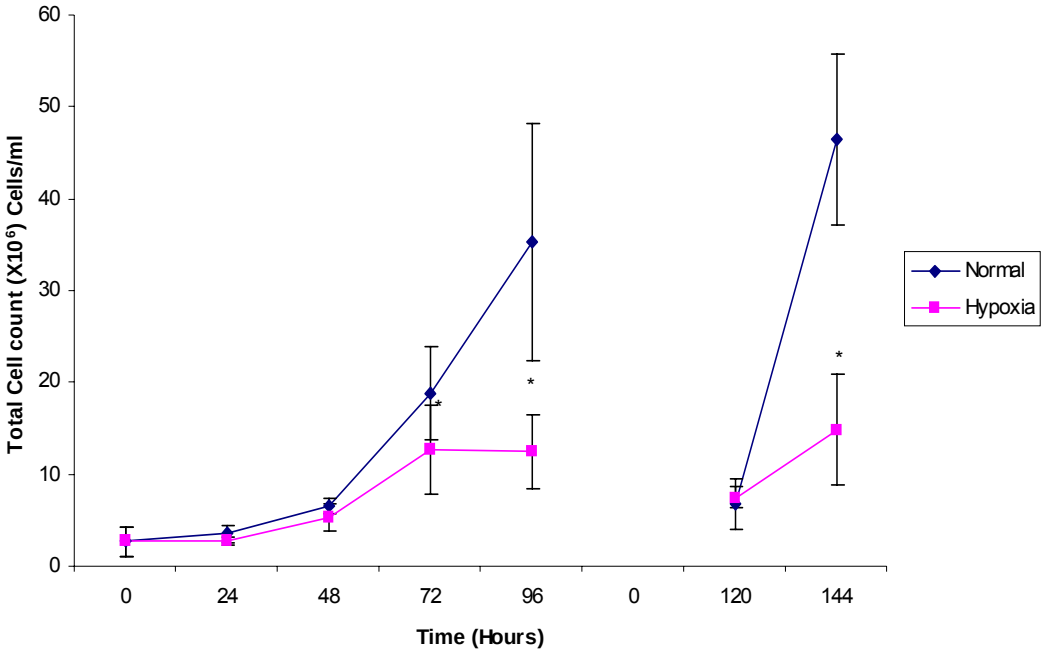
At 72 hours, the normal culture had 18.80±5.09x10^6cells/ml as total cell count, 17.11±4.41 x10^6cells/ml as total viable cells and 91.24±3.77% as percentage viability and 83-95% as percentage total viability range, while the hypoxic cell culture had 12.62±4.90x10^6cells/ml, 9.10±2.62 x10^6cells/ml, 75.32±11.59% and 60-94% as total cell count, total viable cells, percentage viability and percentage viability range, respectively. The difference in the total cell count and total viable cells between the normal and hypoxic cells was statistically significant ($P<0.05$).

At 96 hours of culture, the normal cells had 35.35±12.92x10^6cells/ml, 32.90±12.0x10^6 cells/ml, 93.34±2.3% and 89-95% as total cell count, total viable cells, percentage viability and percentage viability range, respectively, while the B50 cells under hypoxia had 12.46±4.04x10^6cells/ml, 10.90±4.17x10^6cells/ml, 87.70±7.04% and 76-93% as total cell count, total viable cells, percentage viability and range of percentage viability, respectively. The difference in the total cell count and total viable cells between the normal and hypoxic cells was statistically significant ($P<0.05$).

At 120 hours of culture following splitting of cells in culture plates, the normal B50 cells had 6.77±2.67x10^6cells/ml, 6.30±2.58x10^6cells/ml, 92.15±2.77% and 88-94% as total cell count, total viable cells, percentage viability and range of percentage viability, while the B50 cells in hypoxia had 7.50±1.11x10^6cells/ml, 6.07±0.71x10^6 cells/ml, 81.54±4.39% and 76-87% as total cell count, total viable cells, percentage viability and range of percentage viability, respectively. There was a non significant decrease ($P= 0.06$) in the total cell count and a slight increase in the total number of viable cells between the normal and hypoxic cells. The results showed a decrease in total cell count and an increase the total viable cells in the normal cells when compared with the cells in hypoxia. This may be as a result of cells staying in the culture medium for a long period of time without changing the medium resulting in the depletion of the nutrients and build-up of toxic metabolites giving rise to more cellular death but more viable cells in normal than in hypoxic cells. The colour of the culture media would have been changed from purple to light yellow. If the culture medium is changed, the cells would be revived and proliferate with increase in total cell count and total viable cells but if the medium is not changed all the cells would normally die the following day.

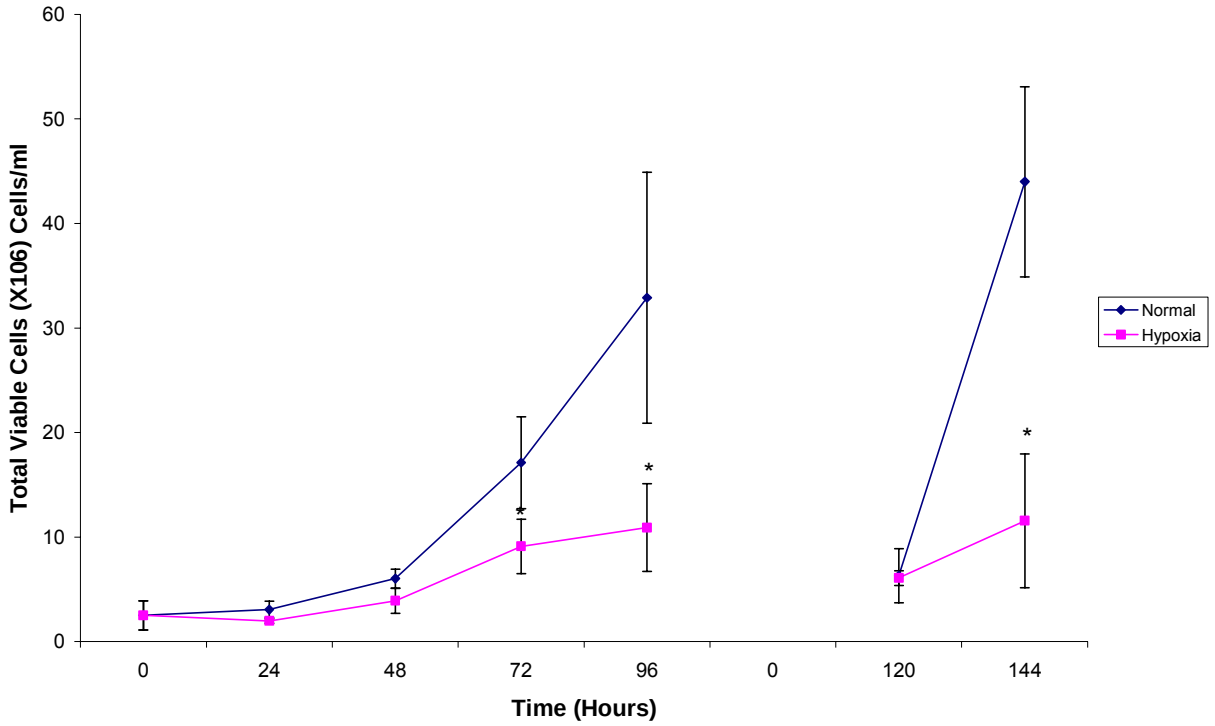
At 144 hours of culture, the normal B50 cells had 46.50±9.32x10^6cells/ml, 44.00±9.10x10^6cells/ml, 94.53±0.8%, and 93-96% as total cell count, total viable cells, percentage viability and percentage viability range, respectively, while those cells cultured under hypoxia had 14.85±6.00x10^6cells/ml, 11.56±6.38x10^6cells/ml, 74.18±13.60% and 56-91% as total cell count, total viable cells, percentage viability and range of percentage viability, respectively, as shown in Figures 3.1; 3.2 and Table 3.1. The difference in the total cell count and total viable cells between the normal and hypoxic cells was significant ($P<0.05$).

Fig.3.1 Time course effects of hypoxia on total cell count of B50 cells in culture



Comparative time effect of hypoxia on total cell count in B50 cells using trypan blue exclusion method (n=6). The cells were cultured in normal (21% O_2; 5% CO_2) and hypoxia (5%O_2; 5%CO_2) conditions. The B50 cells were split at 120 hours represented by split axis (*P<0.05 versus normal; Student's t-test).

Fig.3.2. Time course effects of hypoxia on total viable cell count in B50 cells in culture.



Time effect of hypoxia on total viable cells in B50 cells in culture using trypan blue exclusion method (n= 6). The normal cells cultured in (21% O_2; 5% CO_2) and hypoxic cells (5% O_2; 5% CO_2). The B50 cells were split at 120 hours represented by split axis (*P<0.05 versus normal cells; Student's t-test).

Table 3.1: The time effect of hypoxia on total cell count and viability in B50 cells.

Time(hours)/Type of treatment (n=6)	Total cell count ($x10^6$)cells/ml	Total Viable Cells ($x10^6$)cells/ml	Viability (%)
0 HRS	**2.67 ± 1.64**	**2.5 ± 1.4**	**93.6 ± 2.8**
Range	(1.2-5.0)	(1.1-4.7)	(89.5-97.4)
24 HRS			
NORMAL	**3.5 ±0.9**	**3.05 ±0.8**	**87.1 ± 0.7**
Range	(2.4-4.6)	(2.1-4.0)	(86.1-88.1)
HYPOXIA	**2.8 ± 0.4**	**1.96 ± 0.2**	**70.9 ± 5.4**
Range	(2.1-3.1)	(1.6-2.3)	(63.3-76.2)
48HRS			
NORMAL	**6.5±0.8**	**6.0±0.9**	**92.3±1.2**
Range	(5.2-7.4)	(4.7-6.9)	(90.4-93.6)
HYPOXIA	**5.34±1.45**	**3.9±1.2**	**72.0±3.4**
Range	(3.4-7.5)	(2.3-4.7)	(67.7-76.0)
72HRS			
NORMAL	**18.8±5.1**	**17.1±4.4**	**91.2±3.8**
Range	(10.7-26.8)	(9.4-22.4)	(82.8-95.4)
HYPOXIA	**12.6±4.9***	**9.10±2.62***	**75.3±11.6**
Range	(5.3-21.2)	(4.3-12.8)	(60.4-94.4)
96HRS			
NORMAL	**35.4±12.9**	**32.9±12**	**93.3±2.3**
Range	(16.9-51.0)	(16.1-48.5)	(89.5-95.3)
HYPOXIA	**12.5±4.0***	**10.9±4.2***	**85.7±7.0**
Range	(8.19)	(6-17)	(76.1-93.3)
120HRS			
NORMAL	**6.8±2.7**	**6.3±2.6**	**92.2±2.8**
Range	(3.4-9.9)	(3.0-9.3)	(88.2-94.3)
HYPOXIA	**7.5±1.1**	**6.1±0.7**	**81.5±4.4**
Range	(6.1-8.8)	(5.3-6.7)	(76.1-86.9)
144HRS			
NORMAL	**46.5±9.3**	**44.0±9.1**	**94.5±0.8**
Range	(36.6-57.2)	(34.4-54.2)	(93.7-95.7)
HYPOXIA	**14.9±6.0***	**11.6±6.4***	**74.2±13.6**
Range	(9.1-26.4)	(5.1-24.0)	(55.6-90.9)

Data show a comparative time effect of hypoxia on total cell count, total viable cells and percentage viability in B50 cells in culture using trypan blue exclusion method from three different experiments repeated twice (n=6). The normal cells cultured in (21% O_2; 5% CO_2) and hypoxic cells in (5% O_2; 5% CO_2), showed a significant increase (*$P<0.05$ versus normoxic cells; Student's t-test) in total cell count and total viable cells at 72, 96 and 144 hours. The data expressed as mean ±SD (n=6) and Range.

3.1.5. B50 cell proliferation

The effect of hypoxia on cell proliferation was studied using B50 cells cultured in normal and hypoxic conditions. The cellular proliferation was assayed using Promega cellTiter 96® Plus. Since it has been shown that cellular proliferation is directly proportional to the absorbance at 490nm in the proliferation cellTiter assay method, the time course effect of hypoxia on cellular proliferation, showed that, at 24 hours the absorbance was 1.73±0.61 and 1.78±0.39 for the normal and hypoxic B50 cells respectively, however the difference was not statistically significant (P=0.085). At 48 hours of culture the absorbance was 1.37±0.31 and 1.36±0.21 for the normal and hypoxic cells respectively, which showed no significant difference (P=0.066). At 72 hours of culture the absorbance was 3.50±0.03 and 3.40±0.15 for normal and hypoxic cultures respectively. At 96 hours the absorbance for normal and hypoxic cells were 2.54±0.48 and 2.00±0.25 respectively and the difference was significant ($P<0.05$). At 120 hours of culture, the normal and hypoxic cells had absorbance of 1.53±0.38 and 1.35±0.19 respectively while at of 144 hours of culture the normal and hypoxic cells had absorbance of 1.72±0.40 and 1.24±0.20 respectively of which the level of difference was statistically significant ($P<0.05$).The results are shown in Figures 3.3a, 3.3b and Table 3.2 respectively.

Fig.3.3a.Effect of hypoxia on cell proliferation using absorbance at 490nm of CellTiter Aqueous One soultion assay.

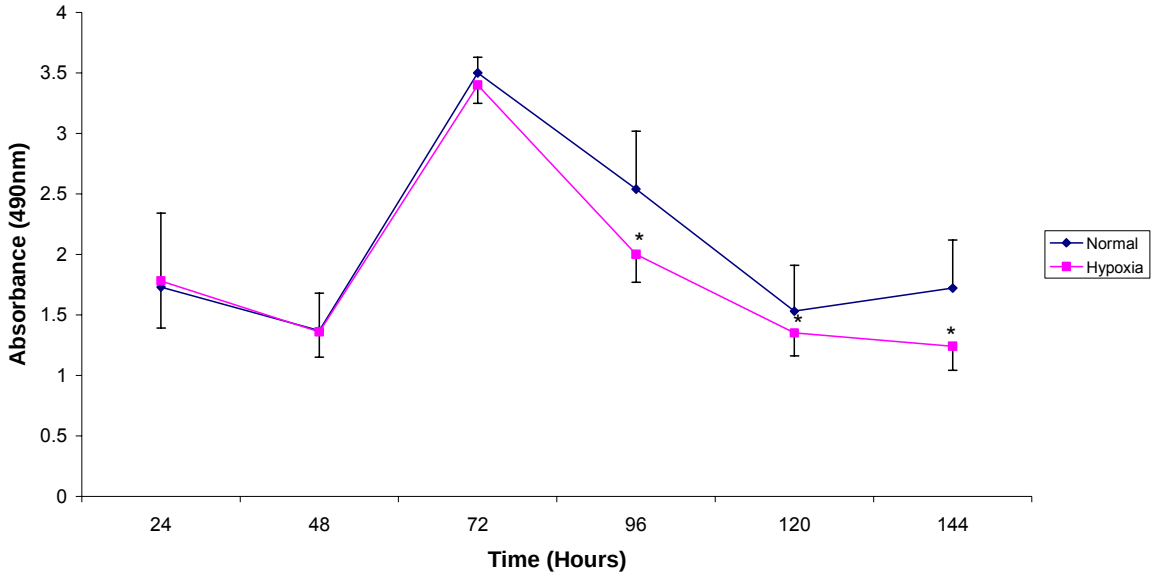
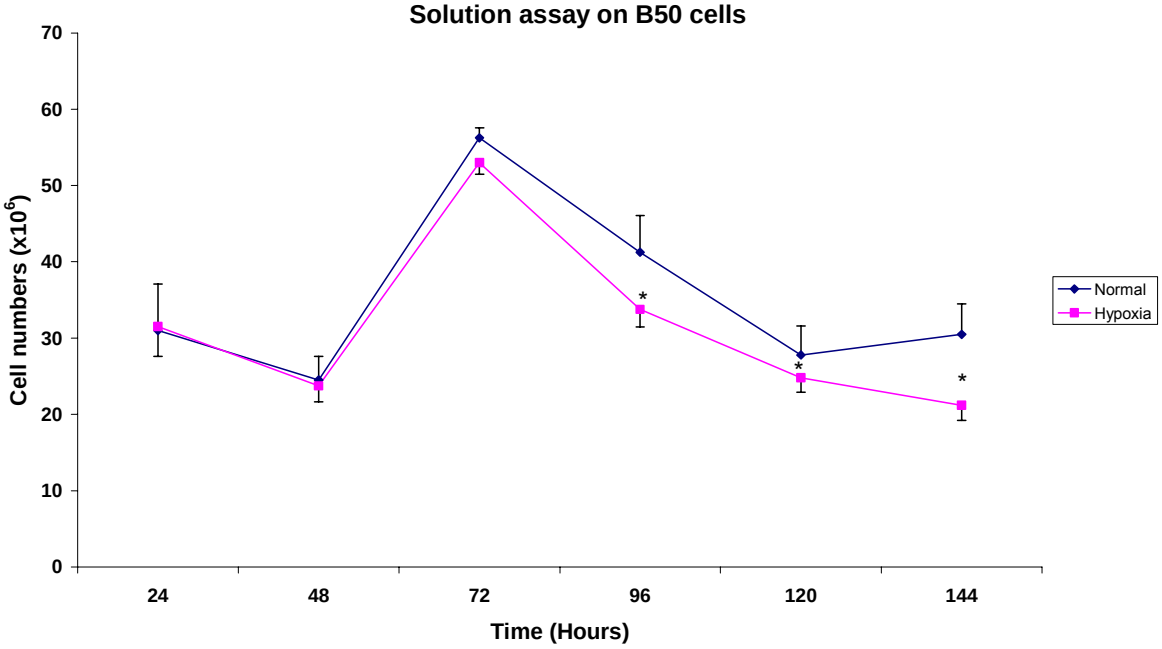


Fig.3.3b. Effect of hypoxia on cell proliferation using CellTiter Aqueous One Solution assay on B50 cells



Figs.3.3a & b. The effect of hypoxia on B50 cell proliferation using CellTiter Aqueous One Solution proliferation assay (a): Since the absorbance from the CellTiter One Solution has been shown to be directly proportional to the number of proliferated cells, the absorbance was plotted against the time of B50 cells in culture. (b): effect on B50 cell proliferation was derived from a standard curve for B50 cells prepared using the trypan blue cell counting method. A plate containing 46.5 x 10^6 cells/ml was prepared and five dilutions (0, 2, 4, 5 and 10) x 10^6 cells/ml were prepared from the 46.5 x 10^6cells/ml. Cells were transferred into microplate and treated with CellTiter and the absorbance at 490nm was measured. The proliferated cells were derived from the standard curve versus the time cell stayed in culture.(n=6; Data as mean ±SD; *P<0.05 versus normal; Student's t-test) normal (21%O_2; 5%CO_2) and hypoxia (5%O_2;5%CO_2).

Table 3.2: The effect of hypoxia on neuronal cell numbers in B50 cells in normal and hypoxic culture.

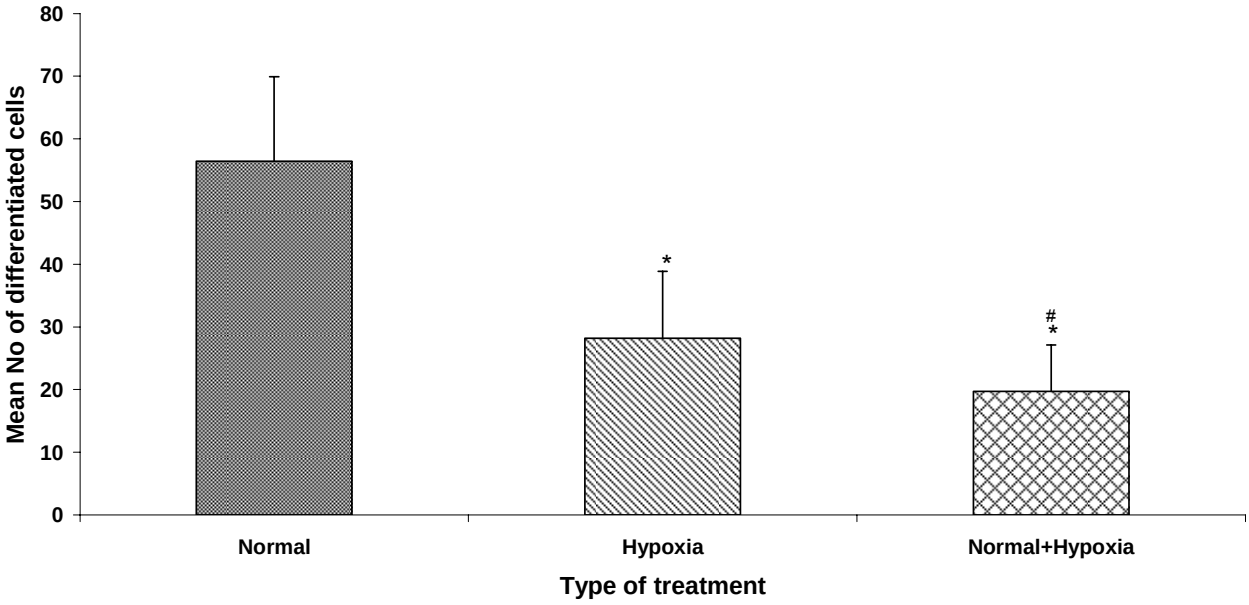
Time (Hours)	Normal			Hypoxia		
	Absorbance	**Cell No ($x10^6$)/ml**	**Rate($x10^6$)/ 24hrs**	**Absorbance**	**Cell No ($x10^6$)/ml**	**Rate ($x10^6$)/ 24hrs**
24	1.73±0.61	31.0	1.29	1.78±0.39	31.5	1.31
48	1.37±0.31	24.50	1.02	1.36±0.21	23.75	0.99
72	3.50±0.03	56.25	2.34	3.40±0.15	53.0	2.20
96	2.54±0.48	41.25	1.72	2.00±0.25*	33.75	1.41
120	1.53±0.38	27.80	1.16	1.35±0.19*	24.80	1.03
144	1.72±0.40	30.50	1.27	1.24±0.20*	21.20	0.88

The effect of hypoxia on B50 cell proliferation was measured as absorbance at 490nm using CellTiter Aqueous One Solution proliferation assay. The cell numbers was derived from the absorbance versus cell number from the standard curve prepared and the daily rate of proliferation was calculated by dividing the number of cells by Time (24hrs). Data is expressed as mean ±SD of the assay (n=6; *P<0.05 versus normal; Student's t-test) normal (21%O_2; CO_2 5%) and hypoxia (5%O_2;5% CO_2).

3.1.6. Neuronal differentiation

The effect of hypoxia on neuronal B50 cell differentiation was studied using DbcAMP to induce differentiation in normal and hypoxic cultures. Neuronal differentiation was assessed by random field strip counting of the differentiated B50 cells, under the microscope at a magnification of 200 times. The differentiated cells counted contained two neuritic processes (axon and dendrite), longer than cell body diameter. The result in Figure 3.4, show that a significantly greater number of B50 cells were differentiated in normal culture compared to those under hypoxia (P<0.05), and compared to those cells that were raised under normal condition but were subjected to hypoxia after 48 hours of normal culture (P<0.05). The extent of neuronal differentiation was affected more in those B50 cells cultured in normal culture and then transferred to hypoxic conditions than those cultured under hypoxia exclusively. The differences in number of differentiated cells was statistically significant (P<0.05).

Fig.3.4.The effect of hypoxia on B50 cells differentiation using DbcAMP and random field assessment

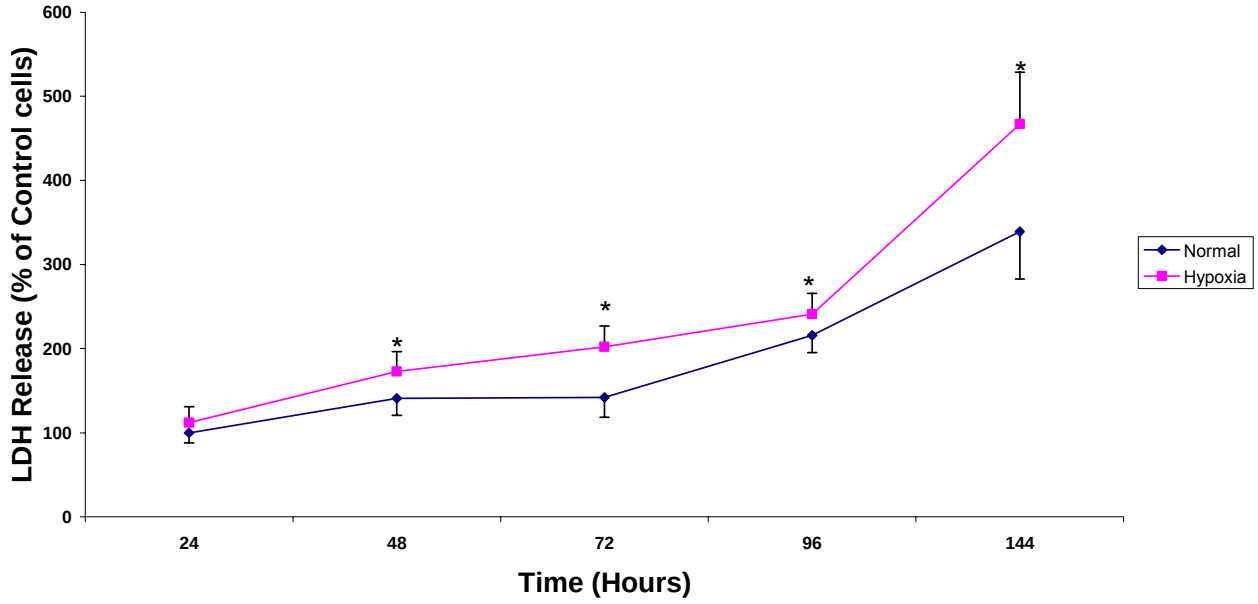


The effect of hypoxia on B50 neuronal cell differentiation using 1mM DbcAMP in culture media and neuronal differentiation was assessed by random field strip counting of the differentiated B50 cells at a magnification x 200. The counted differentiated cells contained an axon and a dendrite longer than cell body diameter and the cells were cultured in Normal ($21\%O_2;5\%CO_2$) and Hypoxia ($5\%O_2;5\%CO_2$) for 96hrs and Normal/Hypoxia; cells were cultured for 48hrs in normal ($21\%O_2;5\%CO_2$) and then transferred to hypoxia ($5\%O_2;5\%CO_2$) for another 48 hrs for a total of 96hr culture. (Data as mean ±SD; n=6; *$P<0.05$ versus normal; $^{\#}P<0.05$ versus hypoxia; Student's t-test).

3.1.7. LDH release from cultured B50 cells

The time-course comparative LDH leakage from normal and hypoxic B50 cells in culture is as shown in Figure.3.5 and Table 3.3. The results show that the greater the time the cells were kept in the culture media, the more LDH was released into the culture media. This occurred in both the normal and hypoxic cultured B50 cells, but the leakage tended to be greater in hypoxic cells than the normal cells, reaching a peak at 144 hours for both normal and hypoxic cells. The result shows that at 24hrs of culture there was no significant difference in LDH release between the hypoxic cells and the normal cells ($P=0.11$), but subsequently there was a significant difference ($P<0.05$) in LDH release between the hypoxic and normal B50 cells in culture.

Fig.3.5.Comparative LDH release from B50 cells cultured in normal and hypoxic conditions



Time-course comparative LDH release from B50 cells cultured in normoxia (21%O_2, 5%CO_2) and hypoxia (5%O_2, 5%CO_2). The LDH released from the normal cells at 24 hrs was used as the control and LDH was expressed relative to the control. The cells were split at 120 hours represented by split line (Data points indicate mean ±SD; n=6;*$P<0.05$ versus normal; Student's t-test).

Table 3.3: Comparative LDH release from normal and hypoxic cells in culture

Time (Hrs)	Normal	% of Control	Hypoxia	% of Control
24	9.92±1.21(Control)	100±12.20	11.12±1.89	112±19.04
48	13.95±2.04	141±20.56	17.18±2.32	173±23.36*
72	14.12±2.32	142±23.39	20.04±2.45	202±24.70*
96	21.41±2.06	216±20.78	23.92±2.47	241±24.89*
144	33.6±5.58	339±56.30	46.31±6.12	467±61.72*

Table shows a time-course comparative LDH release from B50 cells cultured in normoxia (21% O_2, 5%CO_2) and hypoxia (5%O_2, 5%CO_2). Since LDH is present in the normal cells, the LDH released from the 24hrs normal culture was used as a control (100%) for the experiment and the LDH was expressed relative to the control. Cells were split at 120 hours (Data as mean ±SD; n=6; *P<0.05 versus Control; Student's t-test).

3.2 Expression of cannabinoid CB_1 and mu opioid (MOR) receptors in B50 neuronal cells using semi-quantitative RT-PCR

3.2.1 The effect of hypoxia on the expression of CB_1 and MOR receptor mRNA in B50 cells

The result of CB_1 and opioid receptor mRNA expression in B50 neuronal cells in normal, hypoxic and treated cells was studied using reverse transcription polymerase chain reaction (RT-PCR) is shown in Figures 3.6 and 3.7. The results showed that the RT-PCR experiments with the B50 neuronal cells in normal, hypoxic and treated cultures demonstrated positive expression of the cannabinoid CB_1 and mu opioid (MOR) receptors. The mRNA levels of CB_1 and MOR receptors in hypoxic culture of B50 cells were expressed relative to CB_1 and MOR receptors in B50 cells cultured under normal conditions, and these were also compared with mRNA levels of CB_1 and MOR in hypoxic B50 cells treated with different receptor agonists (Figures 3.8 and 3.9). The result showed that there was no significant difference in the level of

CB_1 and MOR receptor mRNA expression between the normal, hypoxic and agonist treated cultured B50 cells.

3.2.2 Semi quantitative RT-PCR of cannabinoid CB_1 and MOR

CB_1 and MOR expression products were subjected to semi quantitative analysis using digital densitometric measurements. The result showed a significant decrease ($P<0.05$) in the density of the RT-PCR products of CB_1 in hypoxic cells treated with 100nM AEA (85%) and 10nM 2-AG (80%) agonists when compared with normal cultured B50 cells (100%), while the increase of 102%; 101%; 107%; 104% and 104% in untreated hypoxic cells and cells treated with 10nM Win, 50nM Win, 10nM AEA and 50nM AEA, were not significantly different from the control (100%). The decrease in the density of CB_1 receptors with 100nM Win (97%); 50nM 2-AG (92%) and 100nM 2-AG (95%), was not significant when compared with the control. The decrease in the density of MOR RT-PCR products was not significant in untreated (MOR) hypoxic cells (93%) and those treated with 10μM DAMGO (98%); 50μM DAMGO and 100μM DAMGO (98%). There was no significant change in densitometric measurements of mRNA area, mRNA volume, height and width respectively when compared with the normal control B50 cells (Tables 3.4A and 3.4B).

When the density of the RT-PCR products was normalised to alpha actin (100%), there was no observed difference in the level of the receptor mRNA expression of CB_1 and MOR in normal, hypoxic and treated cultured B50 cells. The results showed no significant difference between the normal (CB_1) cultured B50 cells (110%), untreated hypoxic cells (112%) and hypoxic treated cells with 10nM Win (111%); 50nM Win (117%); 100nM Win (106%); 10nM AEA 114%; 50nM 2-AG (101%); normal MOR (119%); 10μM DAMGO (118%); 50μM DAMGO (111%) and 100μM DAMGO (118%). Also the CB_1 receptor density showed a non-significant decrease with the cells treated with 100nM AEA (93%) and 10nM 2-AG (83%), when compared to those of alpha actin (100%) (Tables 3.4A , 3.4B and Figures 3.8).

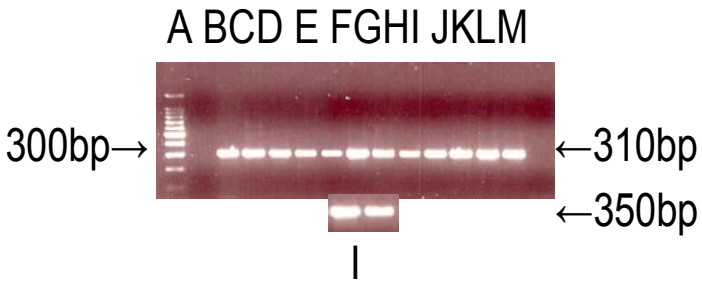


Fig.3.6.The effect of hypoxia and cannabinoid treatment on CB_1receptor expression in B50 cells.

A=DNA Ladder; B=Normal B50 cells; C=Hypoxic B50 cells;

D-I=Hypoxic treated cells with different cannabinoid agonist;

J-M= Hypoxic cells treated cannabinoid agonist/antagonist;

I=Alpha actin

Cannabinoid (CB_1) receptor expression in B50 neuronal cells in culture. Total RNA was extracted from cultured B50 cells in normal (21%O_2, 5%CO_2), hypoxic (5%O_2, 5%CO_2) and hypoxia treated with CB_1 agonists. RT-PCR amplification was performed with specific CB_1 primers. The gel was electrophoresed and stained with ethidium bromide to visualise the intensity of the cDNA for CB_1 (310bp) and was normalised relative to alpha actin (350bp).

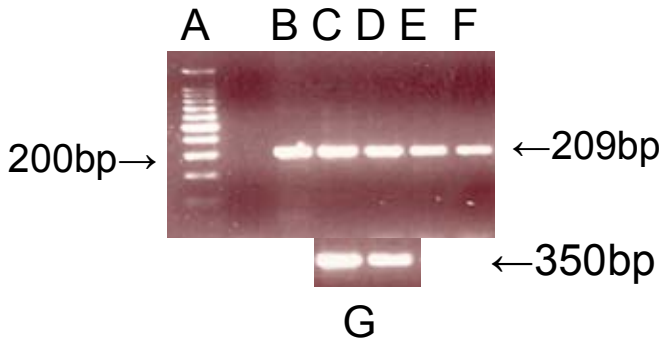


Fig.3.7 The effect of opioid treatment on MOR expression in cultured B50 cells in hypoxia

A=DNA Ladder, B=Normal cells; C=Hypoxic cells;

DEF=Hypoxic MOR treated G=Alpha Actin

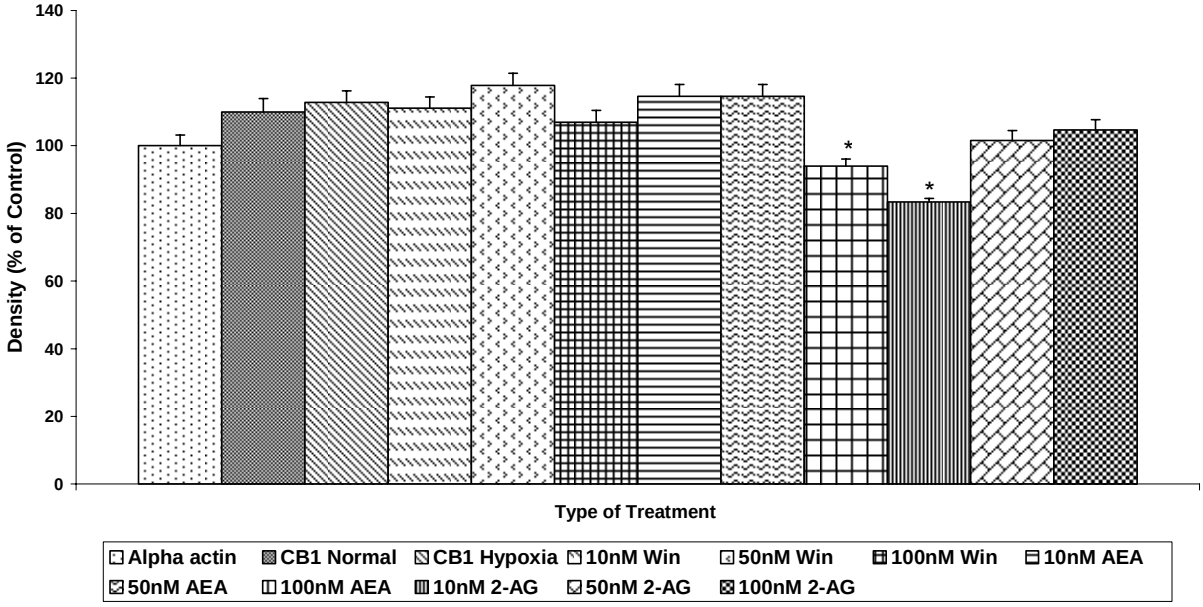
Mu opioid (MOR) receptor expression in B50 neuronal cells in culture. Total RNA was extracted from cultured B50 cells in normal (21%O_2, 5%CO_2), hypoxic (5%O_2, 5%CO_2) and hypoxia treated with MOR agonists. RT-PCR amplification was performed with specific MOR primers. The gel was electrophoresed and stained with ethidium bromide to visualise the intensity of the cDNA for MOR (209bp) and was normalised relative to alpha actin (350bp).

Table 3.4A: Semi-Quantitative RT-PCR product of CB_1 in B50 cells in culture.

Treatment Type	Vol. mm^3	Area mm^2	STD ±	Density	Width	Height	% of Normal	% of Control
CB_1Norma	09.61	4.17	0.39	571.41	1.40	2.98	100	110.0
Hypoxia	15.75	6.66	0.33	586.22	1.78	3.75	102.59	112.85
10nM Win	12.32	5.24	0.33	577.20	1.58	3.36	101.01	111.12
50nM Win	10.59	4.28	0.35	612.24	1.77	2.40	107.15	117.86
100nM Win	6.35	2.83	0.35	555.37	1.21	2.35	97.19	106.91
10nM AEA	10.65	4.44	0.35	595.31	1.40	3.18	104.18	114.60
50nM AEA	5.91	2.60	0.35	595.31	1.40	3.18	104.18	93.98
100nM AEA	3.91	1.98	0.21	488.19	0.76	2.60	85.44*	83.37
10nM 2-AG	6.99	3.53	0.03	459.07	0.38	1.40	80.34*	101.53
50nM 2-AG	8.64	4.06	0.30	527.40	1.21	3.37	92.30	104.65
100nM 2-AG	8.90	4.06	0.31	543.60	1.21	3.37	95.13	104.65
Alpha actin	10.35	3.22	0.32	519.46	2.02	3.12	90.91	100

Cannabinoid CB_1 receptor was expressed in B50 neuronal cells in culture with total RNA extracted from B50 cells in normal ($21\%O_2$, $5\%CO_2$), hypoxic ($5\%O_2$, $5\%CO_2$) and hypoxia treated with CB_1 agonists. The RT-PCR was amplified with specific CB_1 primers and gel was electrophoresed and stained with ethidium bromide to visualise the intensity of the cDNA and normalised relative to alpha actin. The RT-PCR product was quantified with digital densitometer and expressed as percent of control. (Data presented as mean ±SD; *$P<0.05$ versus control; Student's t-test).

Fig. 3.8. The effect of hypoxia on the expression density of CB_1 receptor in B50 cells



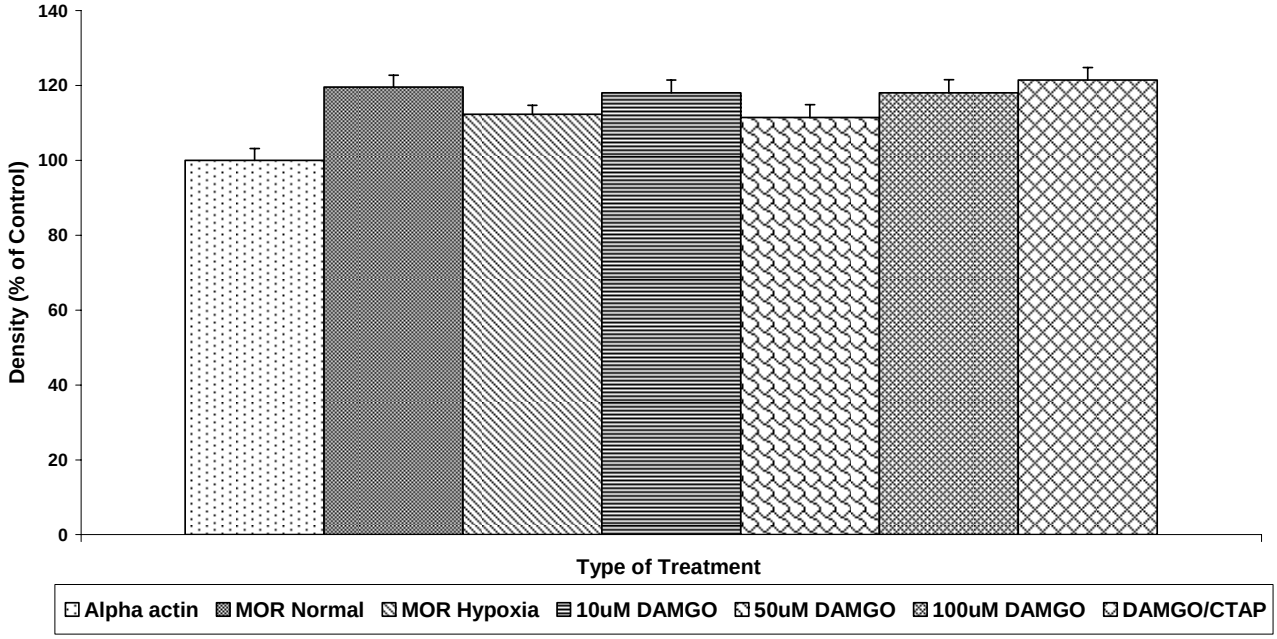
Cannabinoid (CB_1) receptor was expressed in B50 neuronal cells in culture with total RNA extracted from B50 cells in normal ($21\%O_2$, $5\%CO_2$), hypoxic ($5\%O_2$, $5\%CO_2$) and hypoxia treated with CB_1 agonists. The RT-PCR was amplified with specific CB_1 primers and gel was electrophoresed and stained with ethidium bromide and normalised relative to alpha actin. The RT-PCR product was quantified with digital densitometer and expressed as percent of control. (Data presented as mean ±SD; *$P<0.05$ versus control; Student's t-test).

Table 3.4B: Semi-Quantitative RT-PCR product of MOR receptors in B50 cells in culture.

Treatment Type	Vol. mm^3	Area mm^2	STD ±	Density	Width	Height	% of Normal	% of Control
MOR Normal	11.20	4.04	0.32	621.12	2.78	1.56	100	119.57
MOR Hypoxia	04.55	1.94	0.24	583.40	0.76	2.54	93.93	112.31
10µM DAMGO	14.57	5.90	0.35	613.07	2.73	2.16	98.70	118.02
50µM DAMGO	12.48	5.34	0.34	579.21	1.59	3.37	93.27	111.50
100µM DAMGO	10.61	4.29	0.35	613.22	1.78	2.41	98.73	118.05
DAMGO/ CTAP	11.29	4.44	0.33	631.09	2.79	1.59	101.61	121.49
Alpha actin	10.35	3.22	0.32	519.46	2.02	3.12	83.63	100

MOR receptor was expressed in B50 neuronal cells in culture with total RNA extracted from B50 cells in normal ($21\%O_2$, $5\%CO_2$), hypoxic ($5\%O_2$, $5\%CO_2$) and hypoxia treated with MOR agonists. The RT-PCR was amplified with specific MOR primers and gel was electrophoresed and stained with ethidium bromide and normalised relative to alpha actin. The RT-PCR product was quantified with digital densitometer and expressed as percent of control. (Data presented as mean ±SD; Student's t-test).

Fig.3.9. The effect of hypoxia on the expression density of MOR receptor in B50 cells.



MOR receptor was expressed in B50 neuronal cells in culture with total RNA extracted from B50 cells in normal ($21\%O_2$, $5\%CO_2$), hypoxic ($5\%O_2$, $5\%CO_2$) and hypoxia treated with MOR agonists. The RT-PCR was amplified with specific MOR primers and gel was electrophoresed and stained with ethidium bromide and normalised relative to alpha actin. The RT-PCR product was quantified with digital densitometer and expressed as percent of control. (Data presented as mean ±SD; Student's t-test).

3.3. Cannabinoid receptor agonist administration.

3.3.1. The effect of cannabinoid treatment on the morphology of cultured B50 cells

The result of administration of cannabinoid agonists with concurrent exposure of cells to hypoxic conditions, referred to here as pre-treatment against hypoxia, showed B50 cells with fewer aggregates of degenerating and dead B50 cells when compared with hypoxic non-treated B50 cells. The effects of cannabinoid agonist pre-treatment on B50 cells are shown in Plates 9-15. The results showed the effects of pre-treatment with different concentrations of cannabinoid receptor agonists against hypoxia on the B50 neuronal cell morphology as compared to Plates 6-8 for hypoxic un-treated groups. The cells in hypoxic culture without the drug pre-treatment, showed higher incidence of cellular death as evidenced by the number of aggregates of degenerating and dead B50 cells when compared to those cultured under normal condition as shown in Plate 16 and the B50 cells in hypoxia but with cannabinoid agonist pre-treatment as shown in Plates 10-13. The results from the morphological observations showed some changes in the morphology which could have been due to the effects of the different concentrations of the cannabinoid agonists used in the pre-treatments of the B50 cells cultured in hypoxia.

The results of the effect of cannabinoid receptor agonist administration to cells in hypoxic conditions, referred to here as cannabinoid treatment of B50 cells cultured in hypoxia, show cells with fewer aggregates of degenerating and dead cells in cannabinoid treated hypoxic cultures when compared to cultured B50 cells in hypoxia without the cannabinoid treatment (Plates 6-8), while the B50 cells in hypoxia treated with different concentrations of cannabinoid agonists are shown in Plates 17-20. The results of the morphological studies showed that the cannabinoid receptor agonist treatment of the hypoxic B50 cells in culture could have alleviated some of the toxic effects of hypoxia by reducing the number of cellular deaths when compared with cells in hypoxia without treatment. As a result, there was reduced cell death and a healthier cellular morphology in those B50 cells treated with cannabinoid receptor agonists. The results also showed that the cannabinoid treated and pre-treated hypoxic cultured B50 cells were morphologically healthier when compared to the untreated hypoxic cultured B50 cells as shown in the Plates (17-20). The results show that cannabinoid receptor agonists could have some therapeutic and protective benefits

morphologically in hypoxic cultured B50 cells when administered as treatment and pre-treatment.

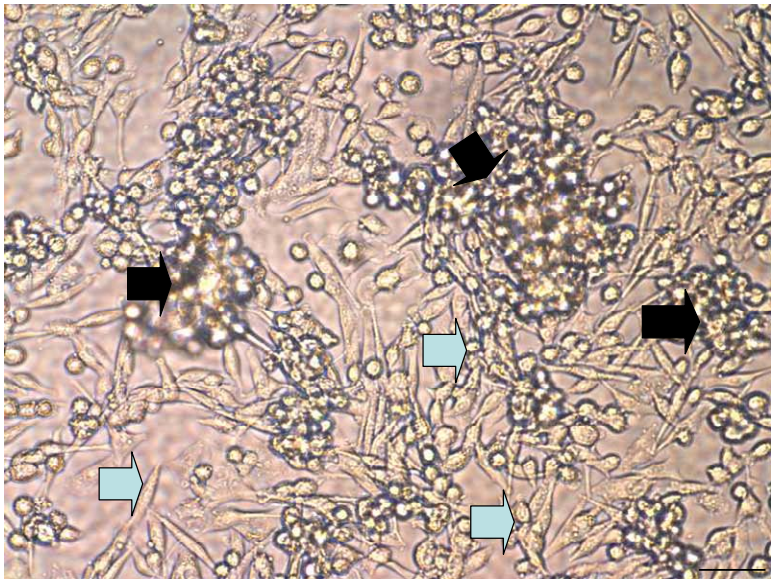


Plate 9:Representative of B50 cells pre-treated against hypoxia with 10nM Win at 0hrs and cultured for 96hrs under hypoxic (5%O_2 and 5% CO_2) conditions, showing some degenerating cells (Black arrow) and some normal cells (Blue arrow). The changes in the B50 cells were observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification

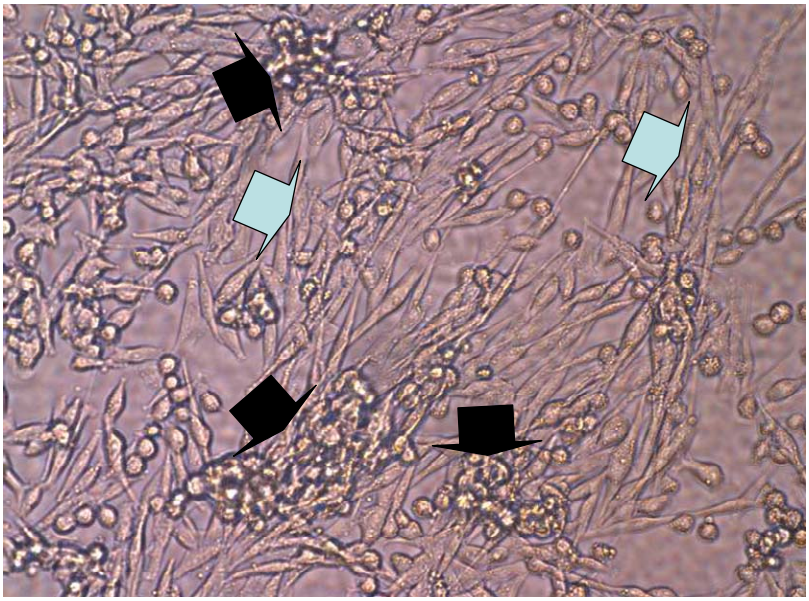


Plate10:Representative of B50 neuronal cells pre-treated against hypoxia with 100nM Win at 0hrs and cultured for 96hrs (5%O2 and 5% CO2), showing few clumps of degenerating cells (Black arrow) and many normal cells (Blue arrow). The changes in the B50 cells were observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

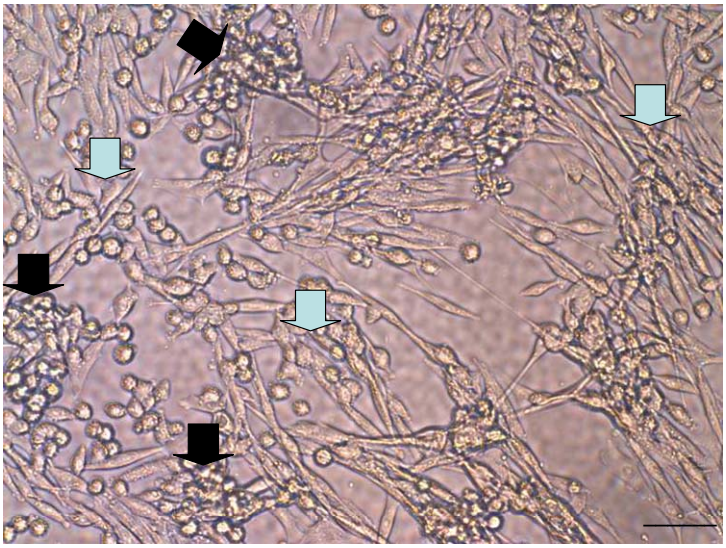


Plate11:Representative of B50 neuronal cells pre-treated against hypoxia with 50nM Win at 0hrs and cultured for 96hrs (5%O_2 and 5% CO_2), showing few degenerating cells (Black arrow) and many normal cells (Blue arrow). The effects in the cells were observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

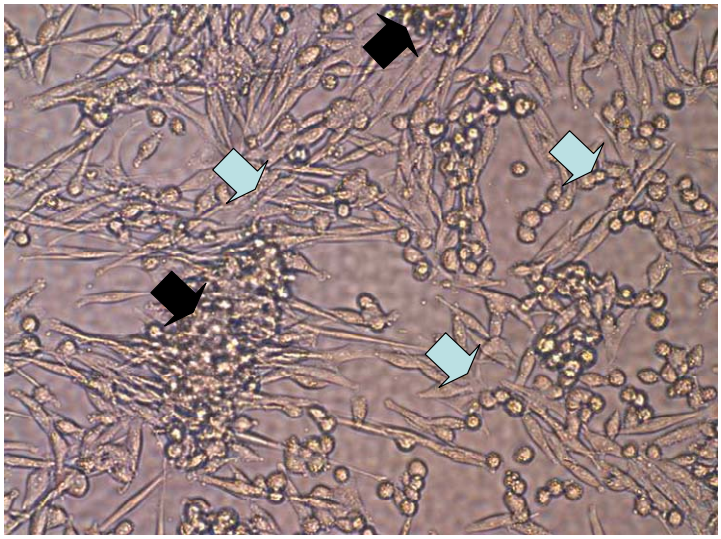


Plate12:Representative of B50 neuronal cells pre-treated against hypoxia with 50nM Win at 0hrs and cultured for 96hrs (5%O2 and 5% CO2), showing few degenerating cells (Black arrow) and many normal cells (Blue arrow). The effects in the cells were observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mmx40 magnification.

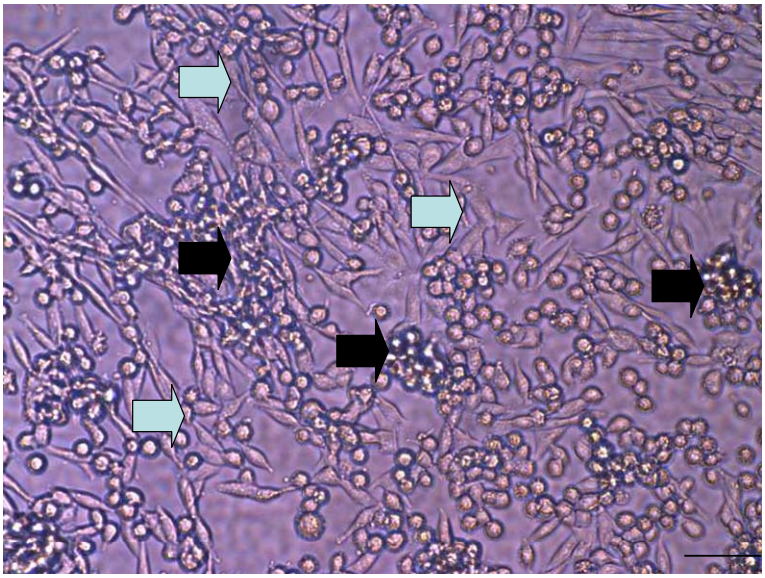


Plate 13:Representative of B50 neuronal cells pre-treated against hypoxia with 50nM 2-AG at 0hrs and cultured for 96hrs (5%O_2 and 5% CO_2),showing many normal cells (Blue arrow) and few degenerating cells (Black arrow). The effects of hypoxia in the cells were observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mmx40 magnification.

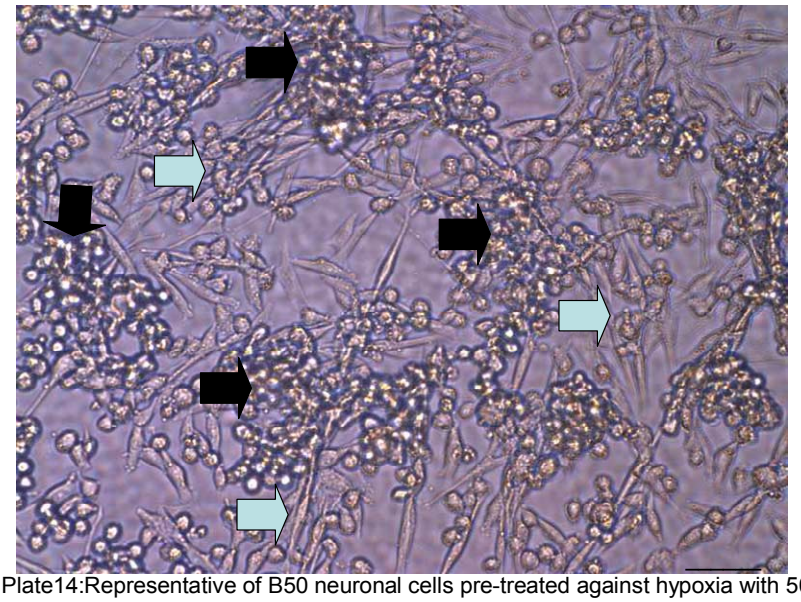


Plate14:Representative of B50 neuronal cells pre-treated against hypoxia with 50nM AEA at 0hrs and cultured for 96hrs (5%O_2 and 5% CO_2), showing some degenerating cells (Black arrow) and few normal cells (Blue arrow). The effects in the cells were observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mmx40 magnification.

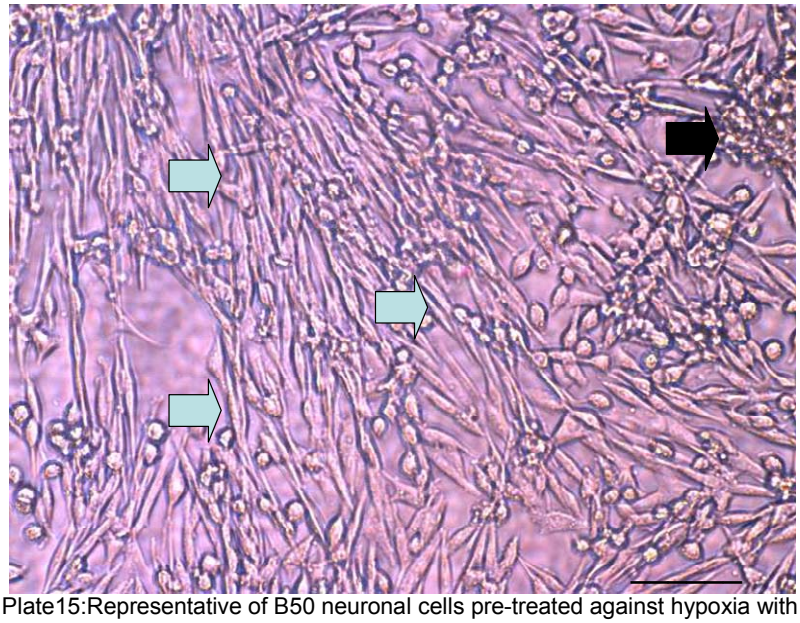


Plate15:Representative of B50 neuronal cells pre-treated against hypoxia with 100nM 2-AG at 0hrs and cultured for 96hrs (5%O_2 and 5% CO_2),showing many normal cells (Blue arrow) and few degenerating cells (Black arrow). The effects in the cells were observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

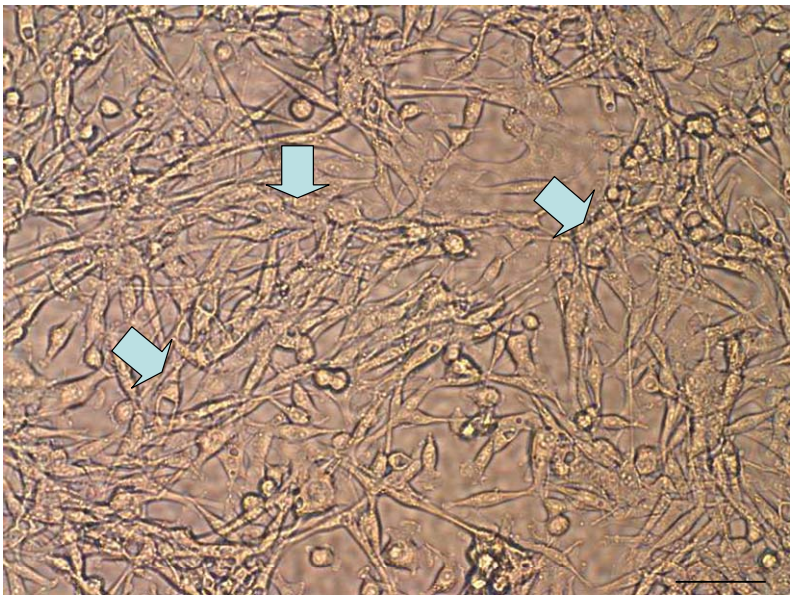


Plate16:Representative of Normal B50 neuronal cells cultured for 96hrs at 21% O_2 and 5% CO_2, showing normal cell morphology (Blue arrow). The cells were observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mmx40 magnification.

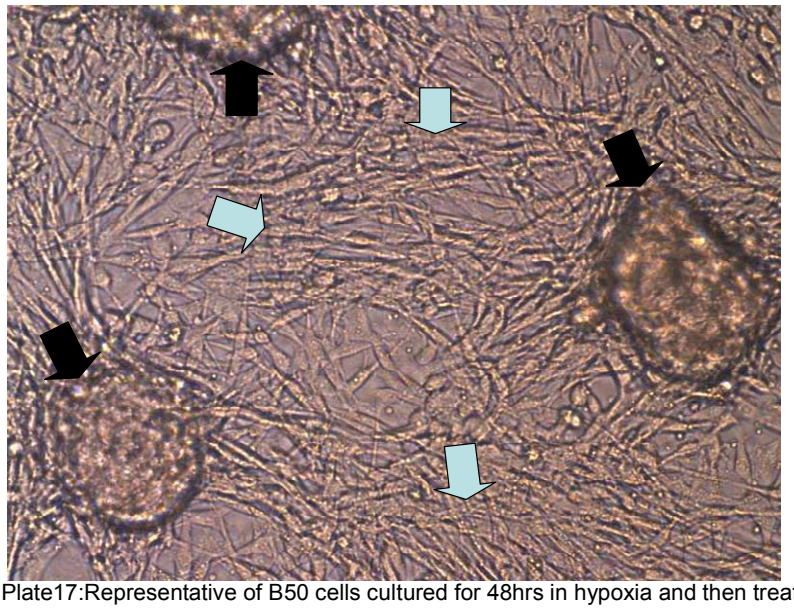


Plate17:Representative of B50 cells cultured for 48hrs in hypoxia and then treated with 100nM Win against hypoxia for 48hrs making up 96hrs of culture in 5%O2 and 5% CO2, showing some groups of degenerating cells (Black arrow) and many healthy cells (Blue arrow). The changes in the cells were observed in six different cultures with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

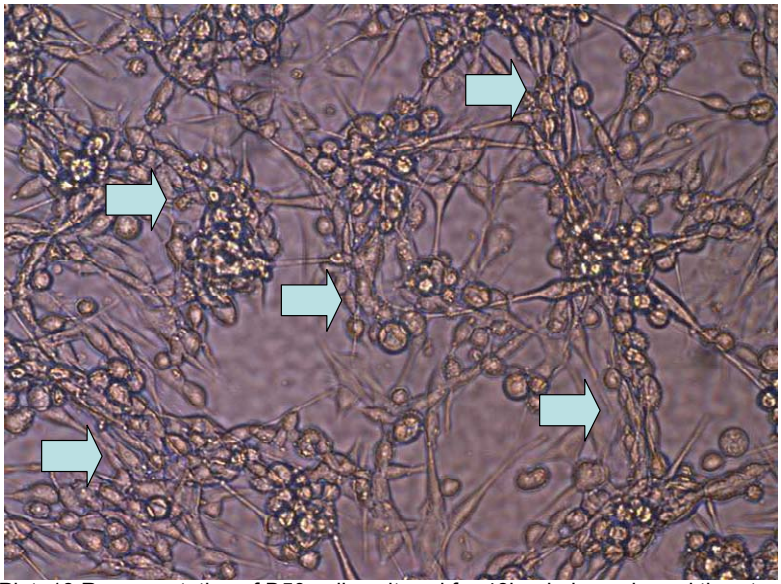


Plate18:Representative of B50 cells cultured for 48hrs in hypoxia and then treated with 10nM Win against hypoxia for 48hrs making up 96hrs of culture in 5%O2 and 5% CO2, showing many normal and healthy cells (Blue arrow). The changes in the cells were observed in six different cultures with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

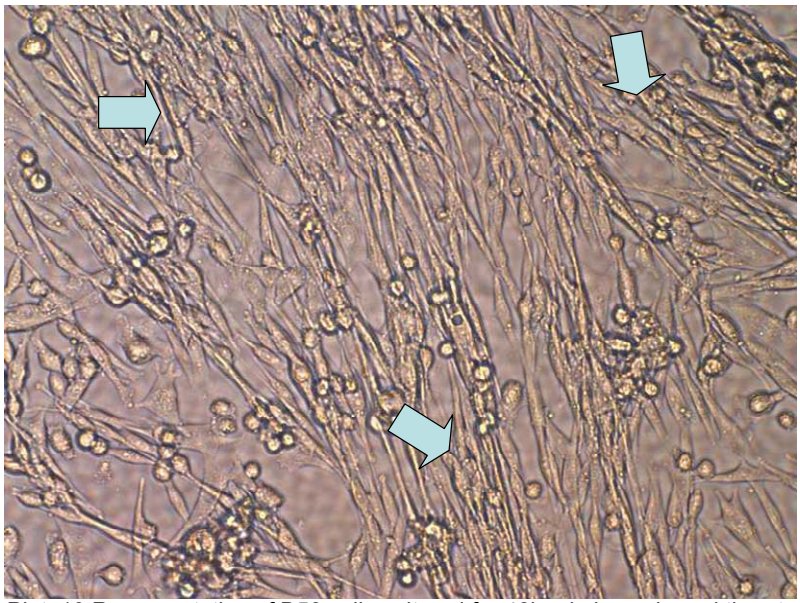


Plate19:Representative of B50 cells cultured for 48hrs in hypoxia and then treated with 50nM Win against the hypoxia for 48hrs making up 96hrs of culture in 5%O2 and 5% CO2. The plate show many normal and healthy cells (Blue arrow). The changes in the cells were observed in six different cultures with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

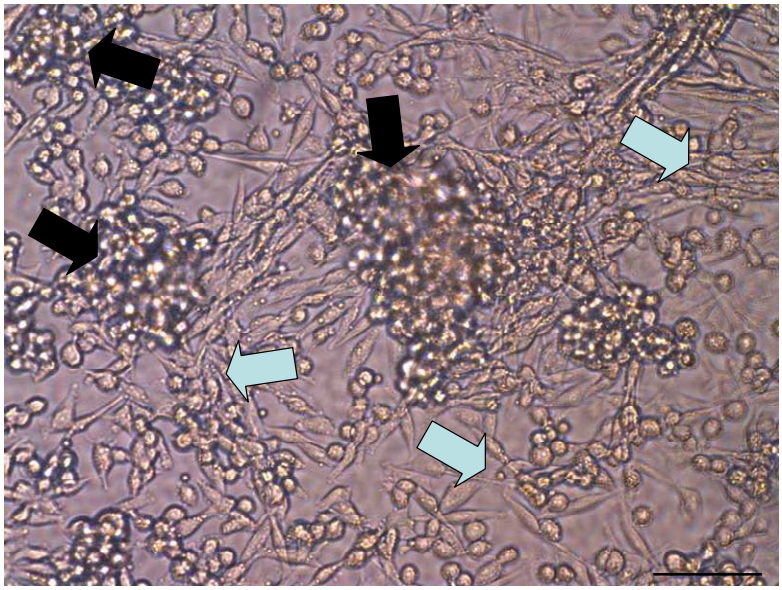


Plate 20:Representative of B50 cells cultured for 48hrs in hypoxia and then treated with 100nM AEA against hypoxia for 48hrs making up 96hrs of culture in 5%O_2 and 5% CO_2), showing some degenerating cells (Black arrow) and some normal cells (Blue arrow). The changes in the cells were observed in six different cultures with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

3.3.2. The effect of cannabinoid agonist treatment on cellular proliferation in normal and hypoxic cultured B50 cells.

The effect of cannabinoid receptor agonist treatment on cellular proliferation in normal cultured neuronal B50 cells is shown in Figure 3.10 and Table 3.5. The result shows an increase in cellular proliferation from the normal cells ($20x10^6$cells/ml) without drug treatment: those cells treated with 10nM Win had $22x10^6$cells/ml, 100nM Win had $24x10^6$cells/ml, 10nM AEA had $25x10^6$cells/ml, 100nM AEA had $25x10^6$cells/ml. Those treated with 10nM 2-AG had $20x10^6$cells/ml and 100nM 2-AG had $17x10^6$cells/ml. The increase in cellular proliferation in B50 cells from the normal was significant ($P<0.05$) with those treated with 100nM Win, 10nM AEA and 100nM AEA, but the decrease in cellular proliferation in cells treated with 100nM 2-AG was not significant ($P=0.21$). The treatment with 10nM 2-AG ($20x10^6$cells/ml) produced no change in the level of cellular proliferation in B50 cells when compared with the normal control B50 cells ($20x10^6$cells/ml). The effect of cannabinoid agonist treatment on cellular proliferation in cultured B50 cells in hypoxia is shown in Figure 3.11 and Table 3.6. The results showed a significant reduction ($P<0.05$) in B50 cell proliferation in hypoxic cells with and without cannabinoid treatment when compared with normal cultured B50 cells with no drug treatment. The results are: normal cells without drug treatment $20x10^6$cells/ml, hypoxic cell with no treatment $7x10^6$cells/ml, 10nM Win $11x10^6$cells/ml, 100nM Win $13.7x10^6$cells/ml, 10nM AEA $10.6x10^6$cells/ml, 100nM AEA $12.2x10^6$cells/ml, 10nM 2-AG $10x10^6$cells/ml and 100nM 2-AG $13.8x10^6$cells/ml. When the results of cellular proliferation in B50 cells were compared between the hypoxic B50 cells without drug treatment ($7x10^6$cells/ml) and the hypoxic B50 cells treated with different concentrations of cannabinoid agonists, the result showed a significant increase ($P<0.05$) in cellular proliferation.

Table 3.5: The effect of cannabinoid agonist treatment on cellular proliferation in normal cultured B50 cells using proliferation assay.

Type of Treatment	Measured Optical Density	Calculated Value ($x10^6$)cells/ml
Normal Cells no Drug	1.5	20.00±0.47
10nM Win	1.6	22.40±1.25*
100nM Win	1.9	24.40±0.70*
10nM AEA	2.0	25.00±0.01*
100nM AEA	2.2	25.25±0.51*
10nM 2-AG	1.5	20.00±0.12
100nM 2-AG	1.3	17.50±0.42

(Data presented as means ±SD; n=6;*P<0.05 versus untreated normal cells; Student's t-test).

Table 3.6: The effect of cannabinoid agonist treatment on cellular proliferation in cultured B50 cells in hypoxia using proliferation assay.

Type of Treatment	Measured Optical Density	Calculated Value ($x10^6$)cells/ml
Normal Cells no Drug	1.5	20.00±0.47*
Hypoxic cells no drug	0.35	7.0±0.12
10nM Win	0.62	11.25±0.24*
100nM Win	0.81	13.70±0.42*
10nM AEA	0.58	10.60±0.04*
100nM AEA	0.74	12.20±0.02*
10nM 2-AG	0.46	10.00±0.04*
100nM 2-AG	0.87	13.80±0.29*

(Data presented as means ±SD; n=6;*P<0.05 versus hypoxic, untreated cells; Student's t-test).

Tables 3.5 and 3.6 show effect of cannabinoid agonist on cell proliferation of B50 cells cultured in normal (21%O_2; 5%CO_2) and hypoxic (5%O_2; 5%CO_2) conditions using proliferation assay method. The cells were cultured for 48hrs and then treated with different cannabinoid agonists at different concentrations for 48hrs i.e. a total of 96hrs of cell culture. The number of proliferated B50 cells was obtained from the standard curve prepare using trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing $42.5x10^6$ cells/ml was used to prepared five dilutions (0, 2, 4, 5 and 10) $x10^6$ cells/ml and the absorbance (n=5) was measured at 490nm and plotted against the number of cells to give the standard curve. The numbers of cells from the test groups were derived by plotting the absorbance of the test groups against the known cell numbers from the standard.

Fig.3.10. The effect of cannabinoid treatment on cellular proliferation in normal cultured B50 cells

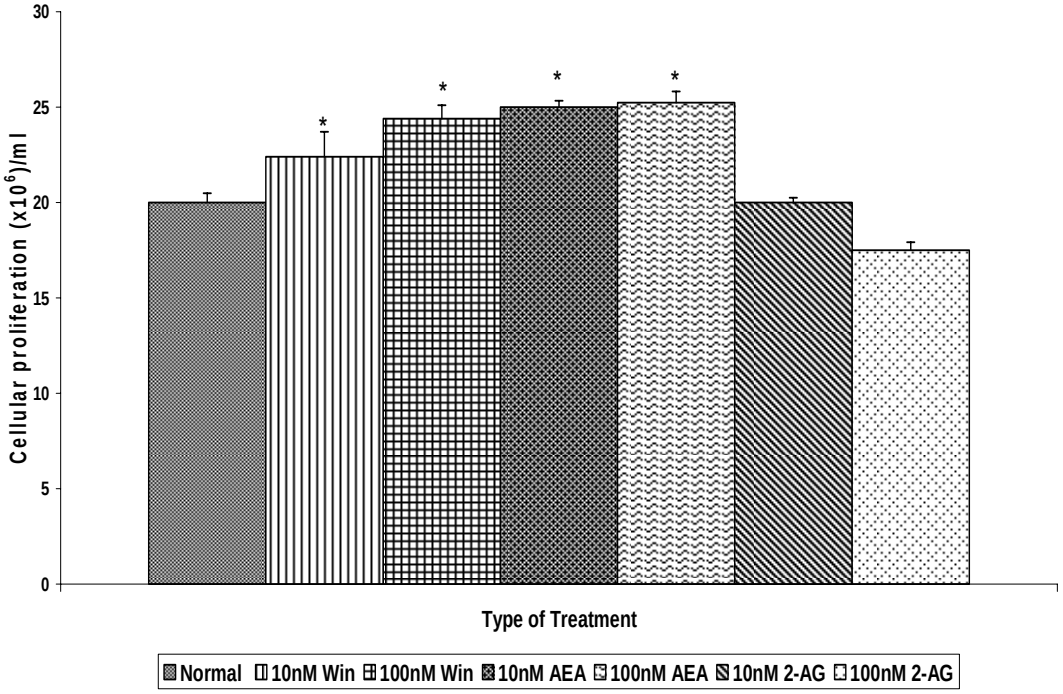


Fig.3.10. Effect of cannabinoid agonist on cell proliferation of B50 cells cultured in normal (21%O_2; 5%CO_2) conditions using proliferation assay method. The cells were cultured for 48hrs and then treated with the cannabinoid agonists for 48hrs, i.e. a total of 96hrs of culture. The number of proliferated cells was quantified using standard curve prepared using trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing 42.5x10^6cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) x 10^6 cells/ml and the absorbance (n=5) was measured at 490nm and plotted against the number of cells to give a standard curve. The number of cells from the tests was quantified using the absorbance of the test groups against the known cell numbers from the standard.(*$P<0.05$ versus normal, untreated cells; Student's t-test).

Fig. 3.11.The effect of cannabinoid agonist treatment on cellular proliferation in cultured B50 cells in hypoxia

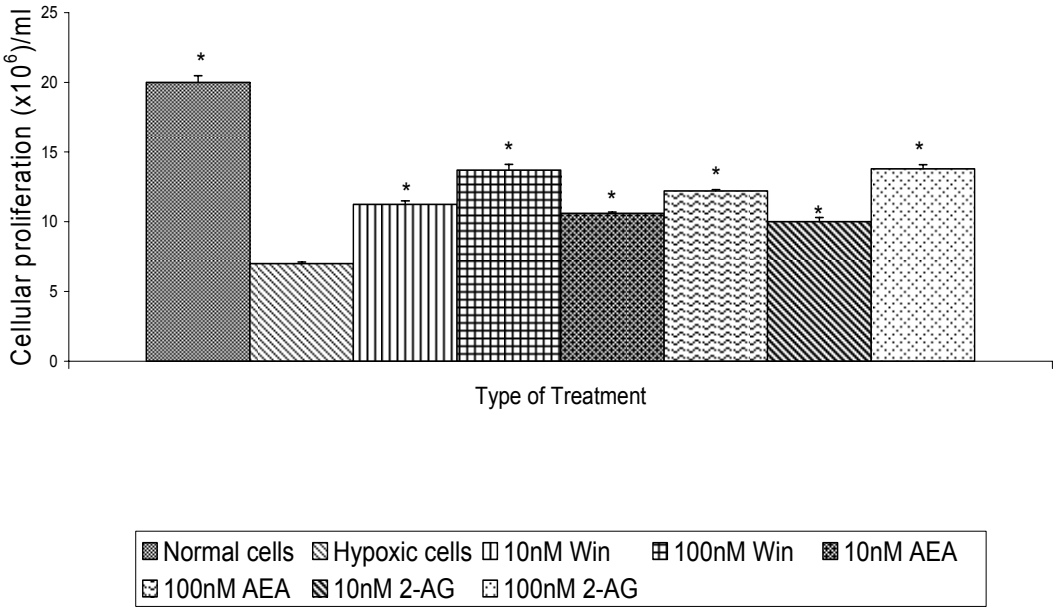


Fig.3.11. Effect of cannabinoid agonist on proliferation of B50 cells cultured in hypoxia (5%O_2; 5%CO_2) using proliferation assay method. The cells were cultured for 48hrs and then treated with the cannabinoid agonists for 48hrs, i.e. a total of 96hrs of culture. The number of proliferated cells was quantified from standard curve prepared using trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing 42.5x10^6cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) x10^6cells/ml and the absorbance (n=5) was measured at 490nm and plotted against the number of cells to give a standard curve. The numbers of cells from the tests were quantified using the absorbance of the test groups against the known cell numbers from the standard. (*$P<0.05$ versus hypoxic untreated cells; Student's t-test).

The effect of cannabinoid agonist concurrent administration at 0hr and culture of B50 cells, referred as pre-treatment on cellular proliferation in normal cultured B50 cells is shown in Figure 3.12. The cells were cultured for 96hrs and the results show that normal cultured B50 cells with drug pre-treatment had either increased or decreased cellular proliferation when compared to the normal untreated B50 cells. The results were normal, untreated cells $20x10^6$cells/ml, 10nM Win $10.9x10^6$cells/ml, 50nM Win, $15.6x10^6$cells/ml; 100nM Win, $13.7x10^6$cells/ml; 10nM AEA, $7.5x10^6$cells/ml; 50nM AEA, $20x10^6$cells/ml; 100nM AEA, $21x10^6$cells/ml; 10nM 2-AG, $20.5x10^6$cells/ml; 50nM 2-AG, $20.5x10^6$cells/ml; 100nM 2-AG, $26.8x10^6$cells/ml. The decrease in cell proliferation was significant ($p<0.05$) between the untreated normal B50 cells and those pre-treated with 10nM Win ($10.9x10^6$cells/ml) and 10nM AEA ($7.5x10^6$cells/ml). The decrease in cell proliferation was not significant ($P=0.63$) between the normal untreated B50 cells and those pre-treated with 50nM Win ($15.6x10^6$cells/ml) and 100nM Win ($13.7x10^6$cells/ml). There was a significant increase ($p<0.05$) in cell proliferation between the normal untreated cells and those pre-treated with 100nM 2-AG ($26.8x10^6$cells/ml) while the slight increase in proliferation in 100nM AEA ($21x10^6$cells/ml) was not significant ($P=0.82$) and those pre-treated with 50nM AEA ($20x10^6$cells/ml), 10nM 2-AG ($20.5x10^6$cells/ml), and 50nM 2-AG (20.5 $x10^6$cells/ml) showed no change in cellular proliferation when compared with the untreated normal cells which served as the control ($20x10^6$cells/ml) (Table 3.7).

The effects of cannabinoid agonist pre-treatment against hypoxia on cellular proliferation in B50 cells cultured in hypoxia are shown in Figure.3.13. The results showed that the untreated normal B50 cells ($20x10^6$cells/ml), had the same proliferation with the B50 cells pre-treated against hypoxia with 10nM Win ($20x10^6$cells/ml). The proliferation of untreated B50 cells in hypoxia ($7x10^6$cells/ml) was significantly increased ($P<0.05$) when compared with the cells pre-treated against hypoxia with 50nM AEA ($10.6x10^6$cells/ml); 100nM AEA ($10.7x10^6$cells/ml); 100nM 2-AG ($10.5x10^6$cells/ml), 50nM Win ($14.8x10^6$cells/ml); 100nM Win ($14.7x10^6$cells/ml); 10nM Win ($20x10^6$cells/ml) and 10nM AEA ($15.6x10^6$cells/ml), while there was no appreciable change in cellular proliferation in cells pre-treated with 10nM 2-AG ($7.5x10^6$cells/ml); 50nM 2-AG ($7.6x10^6$cells/ml), when compared with the untreated hypoxic B50 cells in culture as shown in Table 3.8.

The effects of cannabinoid agonist and antagonist treatment on cellular proliferation in cultured B50 cells in hypoxia are shown in Figure 3.14. The result showed a significant increase ($P<0.05$) in cellular proliferation between the hypoxic untreated B50 cells ($7x10^6$cells/ml), and those treated with 100nM AM251/100nM Win ($20.10x10^6$cells/ml), 50nM AM630/50nM Win ($13.5x10^6$cells/ml) and 50nM AM251/ 50nM Win ($11.25x10^6$cells/ml) and normal untreated B50 cells ($20x10^6$cells/ml), while the increase with 100nM AM630/100nM Win ($9x10^6$cells/ml) was not significant ($P=0.15$)(Table 3.9).

Table 3.7: The effect of cannabinoid agonist pre-treatment on cellular proliferation in normal cultured B50 cells.

Type of Treatment	Measured Optical Density(490nm)	Calculated Value ($x10^6$cells/ml)
Normal Cells no Drug	1.5	20.00±0.47
10nM Win	0.53	10.90±0.02*
50nM Win	1.03	15.60±0.44*
100nM Win	0.82	13.75±0.23*
10nM AEA	0.37	07.50±0.05*
50nM AEA	1.51	20.00±0.11
100nM AEA	1.65	21.25±0.24
10nM 2-AG	1.57	20.50±0.50
50nM 2-AG	1.57	20.50±0.36
100nM 2-AG	2.16	26.88±0.84*

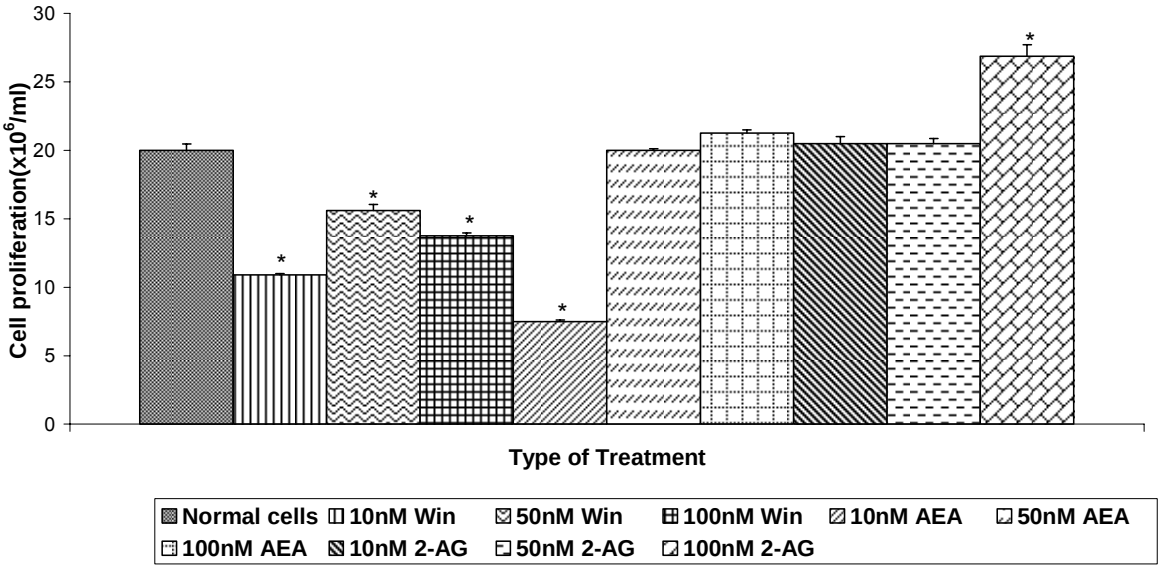
(Data presented as mean ±SD; n=6; *$P<0.05$ versus normal untreated cells; Student's t-test).

Table 3.8: The effect of cannabinoid agonist pre-treatment on cellular proliferation in cultured B50 cells in hypoxia.

Type of Treatment	Measured Optical Density(490nm)	Calculated Value ($x10^6$cells/ml)
Normal Cells no Drug	1.5	20.00±0.47*
Hypoxic cells no drug	0.35	07.00±0.12
10nM Win	1.52	20.10±0.64*
50nM Win	0.94	14.80±0.31*
100nM Win	0.94	14.70±0.16*
10nM AEA	1.03	15.60±0.82*
50nM AEA	0.57	10.60±0.04*
100nM AEA	0.59	10.70±0.28*
10nM 2-AG	0.37	07.50±0.25
50nM 2-AG	0.39	07.60±0.29
100nM 2-AG	0.53	10.50±0.43*

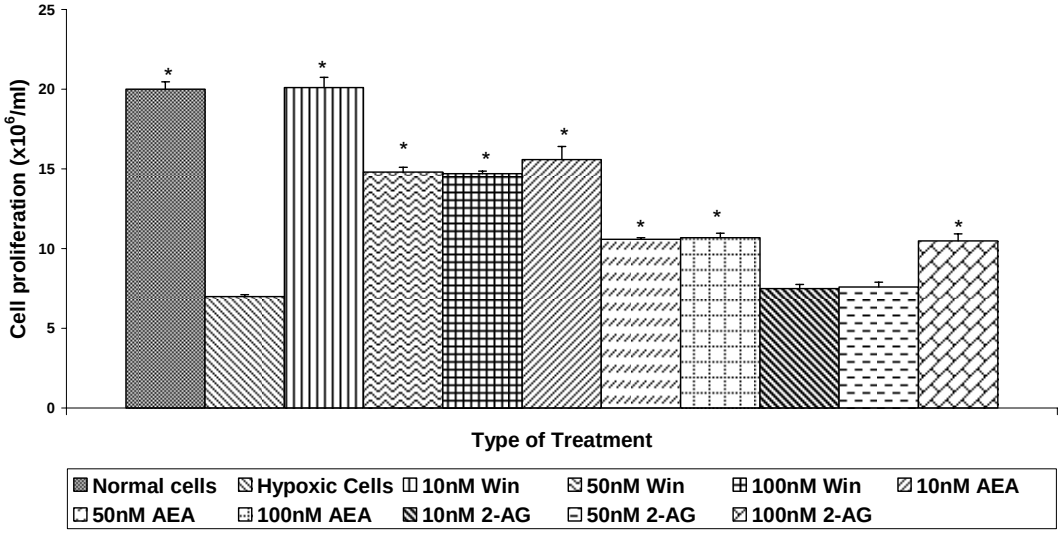
(Data presented as mean ±SD; n=6; *P<0.05 versus hypoxic untreated cells; Student's t-test).

Fig.3.12.The effect of cannabinoid agonist pre-treatment on cellular proliferation in normal cultured B50 cells



The cannabinoid agonist pre-treatment on proliferation of B50 cells in normal ($21\%O_2$; $5\%CO_2$) condition using proliferation assay method. The cells were treated with cannabinoid agonists at 0hr and then cultured for 96hrs. The number of proliferated cells was quantified from standard curve prepared using trypan blue cell counting and CellTiter assay methods. Culture plate containing 42.5 x 10^6cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) x10^6cells/ml and the absorbance at 490nm (n= 5) was plotted against the number of cells. The number of cells from the tests was quantified using the absorbance of the test groups (n=6) against the known cell numbers from the standard.(Data presented as mean ±SD; n=6; *P<0.05 versus untreated normal cells; Student's t-test).

Fig.3.13. The effect of cannabinoid agonist pre-treatment on cellular proliferation in cultured B50 cell in hypoxia



The effect of cannabinoid agonist pre-treatment on proliferation of B50 cells cultured in hypoxic ($5\%O_2$; $5\%CO_2$) conditions using proliferation assay method. The cells were treated with different cannabinoid agonists in different concentrations at 0hr (concurrent treatment) and then cultured for 96hrs in culture. The number of proliferated cells was quantified from standard curve prepared using trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing 42.5 x 10^6cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) x 10^6 cells/ml and the absorbance (n= 5) was measured at 490nm and plotted against the number of cells to give a standard curve. The number of cells from the tests were quantified using the absorbance of the test groups (n=6) against the known cell numbers from the standard.(Data as mean ±SD; n=6; *$P<0.05$ versus hypoxic, untreated cells; Student's t-test).

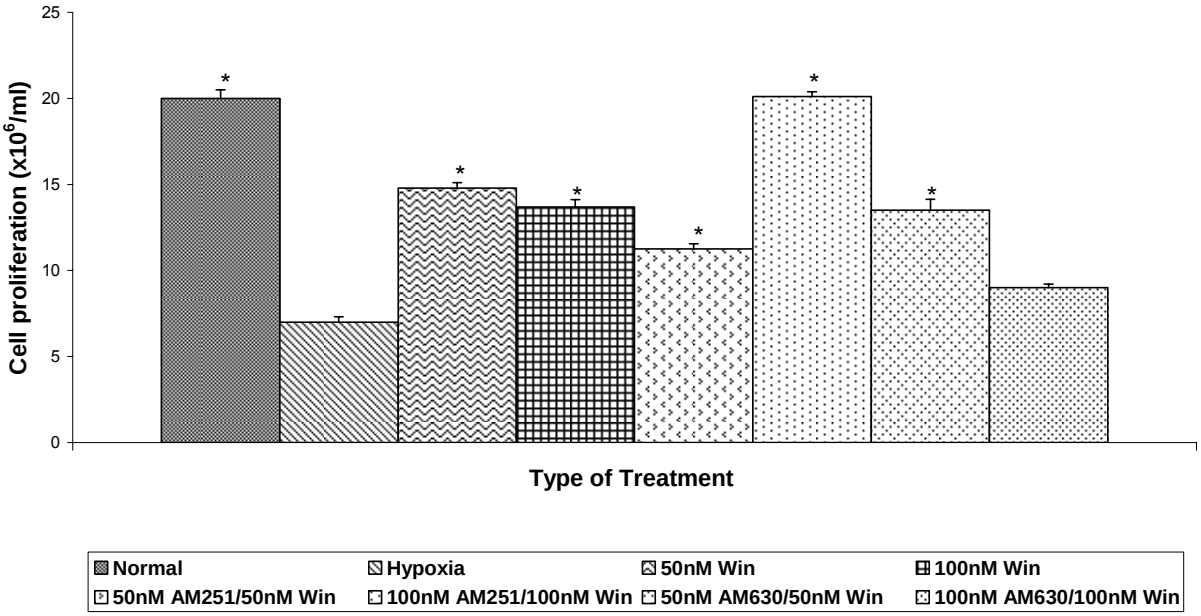
Table 3.9: The effect of treatment of cannabinoid agonist/antagonist on cellular proliferation in cultured B50 cells in hypoxia.

Type of Treatment	Measured Optical Density	Calculated Value ($x10^6$cells/ml)
Normal Cells no Drug	**1.5**	**20.00±0.47***
Hypoxic cells no drug	**0.35**	**07.00±0.12**
50nM Win	**0.94**	**14.80±0.31***
100nM Win	**0.81**	**13.70±0.42***
AM251/50nM Win	**0.69**	**11.25±0.29***
AM251/100nM Win	**1.55**	**20.10±0.03***
AM630/50nM win	**0.86**	**13.50±0.64***
AM630/100nM Win	**0.50**	**09.00±0.11**

(Data presented as mean ±SD; n=6; *P<0.05 versus hypoxic untreated cells; Student's t-test).

The effect of cannabinoid agonist ±antagonist on the proliferation of B50 cells cultured in hypoxia (5%O_2; 5%CO_2), using proliferation assay method. The cells were cultured for 48hrs and then treated with cannabinoid agonists/antagonists for another 48hrs making-up 96hrs of culture. The number of proliferated cells was quantified from the standard curve prepared using trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing 42.5 x 10^6cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) x 10^6cells/ml and the absorbance (n=5) was measured at 490nm and plotted against the number of cells to give a standard curve. The number of cells from the tests were quantified using the absorbance of the test groups (n=6) against the known cell numbers from the standard.(*P<0.05 versus hypoxic, untreated cells; Student's t-test).

Fig.3.14.The effect of cannabinoid agonist/antagonist treatment on cellular proliferation in cultured B50 cells in hypoxia



The effect of cannabinoid agonist±antagonist on the proliferation of B50 cells cultured in hypoxia (5%O_2; 5%CO_2), using proliferation assay method. The cells were cultured for 48hrs and then treated with cannabinoid agonists/antagonists for another 48hrs making-up 96hrs of culture. The number of proliferated cells was quantified from the standard curve prepared using trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing 42.5 x 10^6cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) x 10^6cells/ml and the absorbance (n=5) was measured at 490nm and plotted against the number of cells to give a standard curve. The number of cells from the tests were quantified using the absorbance of the test groups (n=6) against the known cell numbers from the standard.(Data as mean ±SD; *P<0.05 versus hypoxic, untreated cells; Student's t-test).

3.4. LDH release from neuronal B50 cells in culture using LDH assay

3.4.1 LDH release from treatment with cannabinoid agonist/antagonist in B50 neuronal cells cultured in hypoxia.

The LDH release from cultured B50 neuronal cells was used as an index of neuronal injury. LDH release from cultured B50 cells in hypoxia treated with different concentrations of cannabinoid receptor agonists is shown in Figure 3.15. The results show a significant 4-fold increase in LDH leakage in non-treated hypoxic cells (440%), when compared to normal B50 cells (100%) in culture ($p<0.05$). The results of the treatment with different concentrations of cannabinoid agonists, show that cannabinoid agonists have a significant 2-to 4-fold decrease in LDH release from B50 cells treated in hypoxia when compared to the untreated hypoxic B50 cells (440%) ($p<0.05$). The results are 10nM Win (217%), 50nM AEA (181%), 100nM AEA (69%) 50nM 2-AG (209%) and 100nM 2-AG (103%). (Table 3.10).

The results in Figures 3.16a and 3.16b, show the B50 cells treated with cannabinoid agonist in the presence or absence of antagonists. The results show a significant 4-fold increase in LDH released from B50 cells treated with 10nM AM251/10nM Win (840%) when compared to the cells treated with 10nM Win (217%) alone (($p<0.05$), while there was a 2-to 3-fold increase in LDH release from cells treated with 50nM AM630/50nM AEA (212%) and 100nM AM630/100nM AEA (236%) when compared to the cells treated with the cannabinoid agonist alone: 50nM AEA (181%) and 100nM AEA (69%) (Table 3.11).

The LDH release from normal cultured B50 cells treated with cannabinoid agonists is shown in Figure 3.17. The results show that the LDH released in those cells treated with 10nM Win (49%), 100nM Win (31%), and 50nM AEA (57%), 50nM Win (61%), 10nM AEA (59%), 100nM AEA (60%), 50nM 2-AG (59%), was significantly decreased ($P<0.05$) when compared with that from the normal cultured cells without treatment (100%), while the decrease in LDH release was not significant in those B50 cells treated with, 10nM 2-AG (81%) ($P=0.92$) and 100nM 2-AG (66%) ($P= 0.24$), as shown in Table 3.12.

Table 3.10: The effect of cannabinoid agonist treatment on LDH release in cultured B50 cells in hypoxia.

Type of Treatment	Measured Optical Density(OD)	Percent of Control (% of Control)
Normal culture	0.207±0.101	100±10.1
Hypoxia no Drug	0.911±0.211	440±21.1*
10nM Win	0.450±0.102	217±10.2*
50nM Win	0.564±0.112	273±11.2*
100nM Win	1.090±0.225	527±22.5*
10nM AEA.	0.932±0.323	450±32.3*
50nM AEA.	0.374±0.125	181±12.5*
100nM AEA.	0.142±0.101	69±10.1*
10nM 2-AG.	0.482±0.221	233±22.1*
50nM 2-AG.	0.432±0.201	209±20.1*
100nM 2-AG.	0.214±0.111	103±11.1

The effect of cannabinoid agonist treatment on LDH release in B50 cells in hypoxia ($5\%O_2$; $5\%CO_2$), using LDH assay. Cells were cultured for 48hrs in hypoxia and then treated for 48hrs in hypoxia making 96hrs of culture and the absorbance (n=6) was measured at 490nm. The LDH released from the normal culture ($21\%O_2$; $5\%CO_2$), was used as the control (100%) for the experiment and LDH release was expressed relative to the control. (Data as mean ±SD; *$P<0.05$ versus normal untreated cells; Student's t-test).

Table 3.11: The effect of cannabinoid agonist/antagonist treatment on LDH release in cultured B50 cells in hypoxia.

Type of Treatment	Measured Optical Density(OD)	Percent of Control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	0.911±0.211	440±21.1
10nM Win	0.450±0.102	217±10.2
10nmAM251/ 10nM Win	1.738±0.451	840±45.1*
50nM Win	0.564±0.112	273±11.2
50nM AM251/ 50nM Win	0.464±0.251	224±25.1
100nM Win	1.090±0.225	527±22.5*
100nM AM251/ 100nM Win	0.848±0.336	410±33.6
10nM AEA	0.932±0.323	450±32.3
10nM AM630/ 10nM AEA	1.202±0.455	581±45.5*
50nM AEA	0.374±0.125	181±12.5
50nM AM630/ 50nM AEA	0.438±0.321	212±32.1
100nM AEA	0.142±0.101	69±10.1
100nM AM630/ 100nM AEA	0.488±0.333	236±33.3*

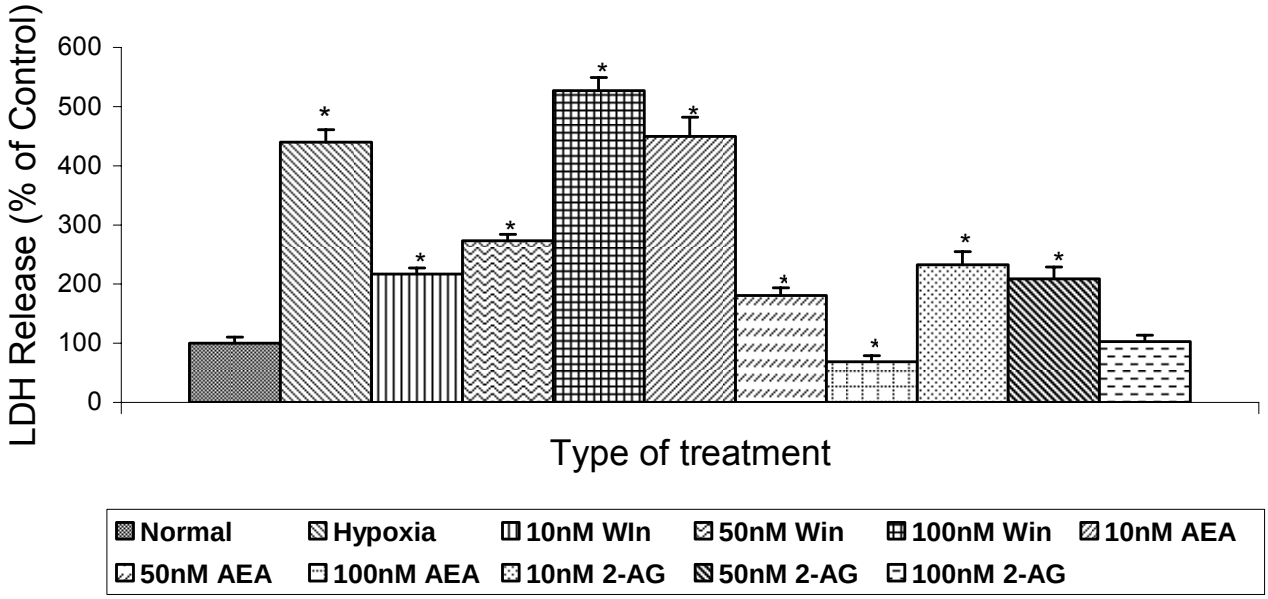
The effect of cannabinoid agonist treatment in the presence of antagonist on LDH release in B50 cells in hypoxia (5%O_2; 5%CO_2), using LDH assay. Cells were cultured for 48hrs in hypoxia and then treated with cannabinoid agonist and equivalent concentration of antagonist for 48hrs in hypoxia making 96hrs of culture and the absorbance (n=6) was measured at 490nm. The LDH released from the normal culture (21%O_2; 5%CO_2), was used as the control (100%) for the experiment and the LDH release was expressed relative to the control. (Data presented as mean ±SD; *$P<0.05$ versus agonist/antagonist treated hypoxic cells; Student's t-test).

Table 3.12: The effect of cannabinoid agonist treatment on LDH release in cultured normal B50 cells.

Type of Treatment	Measured Optical Density(OD)	Percent of Control (% of Control)
Normal no Drug	0.172±0.122	100±12.2
10nM Win	0.083±0.055	49±5.5*
50nM Win	0.104±0.095	61±9.5*
100nM Win	0.053±0.051	31±5.1*
10nM AEA	0.101±0.099	59±9.9*
50nM AEA	0.098±0.089	57±8.9*
100nM AEA	0.103±0.101	60±10.1*
10nM 2-AG	0.139±0.110	81±11.0
50nM 2-AG	0.101±0.100	59±10.0*
100nM 2-AG	0.112±0.102	66±10.2

The effect of cannabinoid agonist treatment on LDH release in normal cultured B50 cells (21%O_2; 5%CO_2), using LDH assay. Cells were cultured for 48hrs in normal culture and then treated for 48hrs in normal conditions making 96hrs of culture and the absorbance (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells, was used as the control (100%) for the experiment and LDH release was expressed relative to the control. (Data presented as mean ±SD; *$P<0.05$ versus untreated normal cells; Student's t-test).

Fig.3.15.The effect of cannabinoid treatment on LDH release from cultured B50 cells in hypoxia.



The effect of cannabinoid agonist treatment against hypoxia on LDH release in B50 cells in hypoxia (5%O_2; 5%CO_2), using LDH assay. Cells were cultured for 48hrs and treated for 48hrs i.e. total of 96hrs of culture. The absorbance (n=6) was measured at 490nm. The LDH released from the normal untreated culture (21% O_2; 5% CO_2) was used as the control (100%) and LDH released was expressed relative to the control (*P<0.05 versus normal; Student's t-test).

Fig.3.16a.The effect of cannabinoid agonist/antagonist treatment on LDH release in cultured B50 cells.

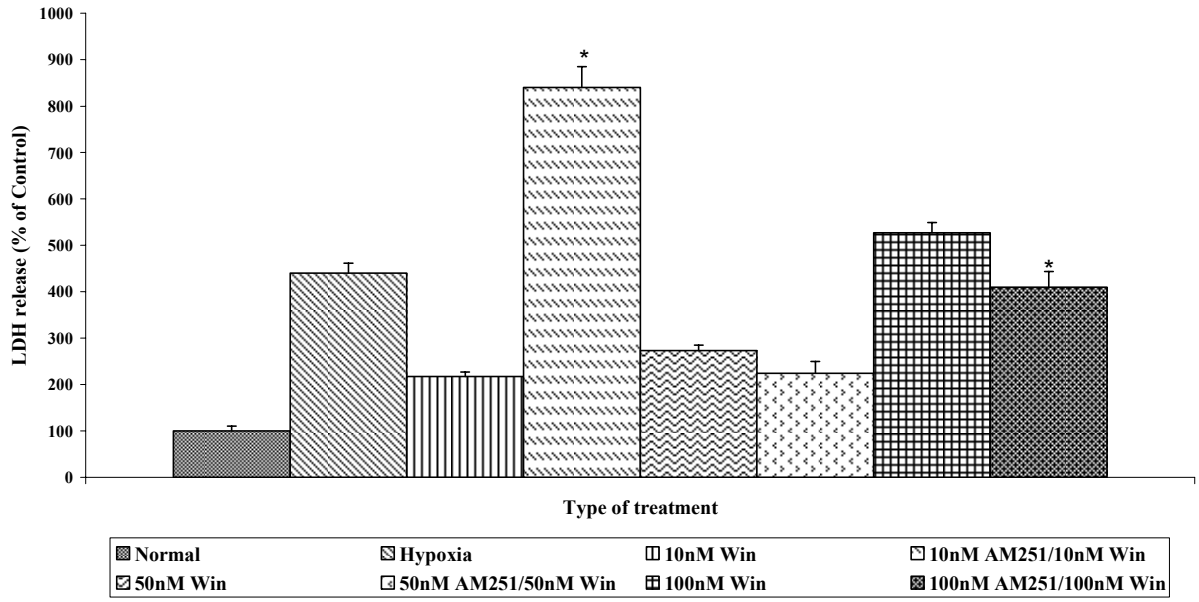
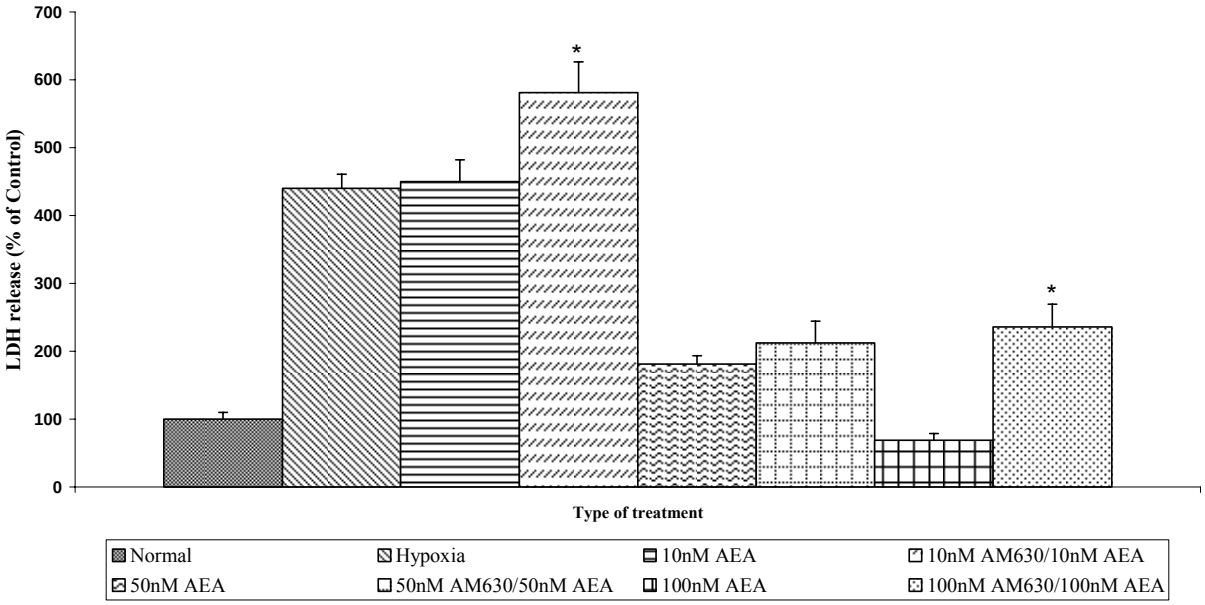
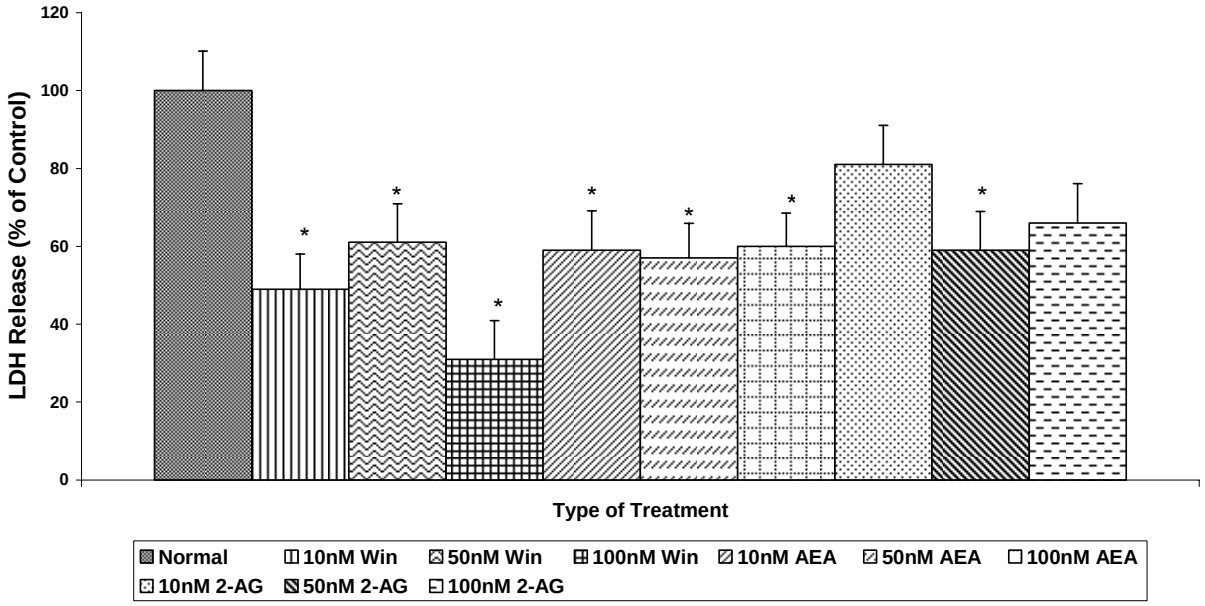


Fig.3.16b.The effect of cannabinoid agonist/antagonist treatment on LDH release in cultured B50 cells



Figs.3.16a&b:The effect of cannabinoid agonist treatment against hypoxia in the presence or absence of antagonist on LDH release in B50 cells in hypoxia (5%O_2; 5%CO_2), using LDH assay. Cells were cultured for 48hrs and then treated with agonist and equivalent concentrations of antagonist for another 48hrs for a total of 96hrs of culture in hypoxia. The absorbance (n=6) was measured at 490nm. The LDH released from the normal untreated culture (21%O_2; 5%CO_2) was used as the control (100%) and LDH released was expressed relative to the control.(*$P<0.05$ versus normal; $^{\#}P<0.05$ versus hypoxic untreated cells; Student's t-test).

Fig.3.17.The effect of cannabinoid treatment on LDH release in normal cultured B50 cells



The effect of cannabinoid agonist treatment on LDH release in normal cultured B50 cells (21%O_2; 5%CO_2), using LDH assay. Cells were cultured for 48hrs in normal culture and then treated with cannabinoid agonists for 48hrs in normal conditions to make-up 96hrs of culture and the absorbance (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells, was used as the control (100%) for the experiment and LDH release was expressed relative to the control. (*$P<0.05$ versus untreated normal cells; Student's t-test).

3.4.2. LDH release from pre-treatment with cannabinoid agonist/antagonist in cultured B50 cells in hypoxia.

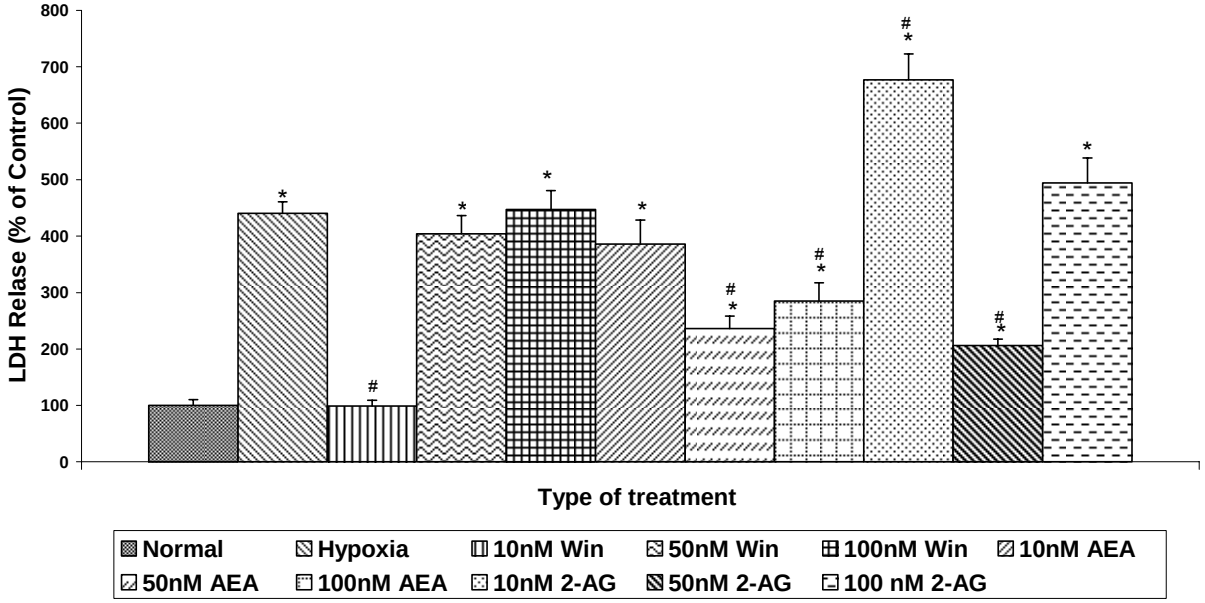
The LDH release from cultured B50 neuronal cells pre-treated against hypoxia with cannabinoid agonists, show that the LDH released was significantly increased in untreated hypoxic cells (440%) and cells pre-treated with various concentrations of cannabinoid agonists ($p<0.05$) when compared to the normal cultured B50 cells (100%), except for the cells pre-treated with 10nM Win (99%). The results show that the LDH leakage from untreated B50 cells in hypoxia (440%), and cells pre-treated with 50nM Win (404%) and 100nM Win (447%); 10nM AEA (386%); 50nM AEA (236%);100nM AEA (285%); 10nM 2-AG (677%), 50nM 2-AG (206%) and 100nM 2-AG (494%), produced increases in LDH release ranging from approximately 2- to 7- fold higher than the level released from normal B50 cells (100%). The results are shown in Figure 3.18. When the LDH release from the untreated B50 cells in hypoxia (440%) was compared with cells pre-treated with cannabinoid agonists, a significant 2- to 4-fold decrease ($p<0.05$) in LDH release was observed in those pre-treated with 10nM Win (99%), 50nM AEA (236%), 100nM AEA (285%) and 50nM 2-AG (206%), and a 2-fold increase in LDH release from cells pre-treated with 10nM 2-AG (677%) when compared with untreated B50 cells in hypoxia (440%) (Table 3.13).

The LDH release from cultured B50 neuronal cells pre-treated against hypoxia with different concentrations of cannabinoid agonist/antagonist is shown in Figures 3.19a and 3.19b. The results showed a significant 2-fold increase in LDH release from cells pre-treated with 10nM AM251/10nM Win (193%), when compared to cells with 10nM Win (99%), a 1.5-fold increase in LDH release from 50nM AM251/50nM Win (550%) when compared to cells with 50nM Win, a 4-fold increase in LDH release from 10nM AM630/10nM AEA (816%) when compared with 10nM AEA (386%) and a 3-fold increase from 100nM AM630/100nM AEA (551%) when compared to cells with 100nM AEA (286%) ($P<0.05$) (Table 3.14).

The effect of cannabinoid agonist pre-treatment on LDH leakage in normal cultured B50 cells is shown in Figure 3.20. The results show that the LDH released in normal cells pre-treated with 10nM Win (54%), 50nM AEA (54%) and 100nM AEA (50%), 50nM Win (58%), 100nM Win (58%), 10nM AEA (62%), and 50nM 2-AG (61%), was significantly reduced ($P<0.05$) when compared with those from normal cultured

B50 cells (100%) without drug pre-treatment, while the decrease in LDH release from those cells pre-treated with 10nM 2-AG (74%) (P=0.21) and 100nM 2-AG (71%) (P=0.54), was not significant (Table 3.15).

Fig.3.18.The effect of cannabinoid pre-treatment on LDH release from cultured B50 cells in hypoxia



The effect of cannabinoid agonist pre-treatment against hypoxia on LDH release in B50 cells in hypoxic condition ($5\%O_2;5\%CO_2$), using LDH assay. Cells were concurrently cultured and treated with cannabinoid agonists at 0hr and then cultured for a total of 96hrs. The absorbance (n=6) was measured at 490nm. The LDH released from the normal untreated culture ($21\%O_2;5\%CO_2$) was used as the control (100%) and results expressed relative to the control. (*$P<0.05$ versus normal; $^{\#}P<0.05$ versus hypoxic untreated cells; Student's t-test).

Fig.3.19a.The effect of cannabinoid agonist/antagonist pre-treatment on LDH release in cultured B50 cells in hypoxia

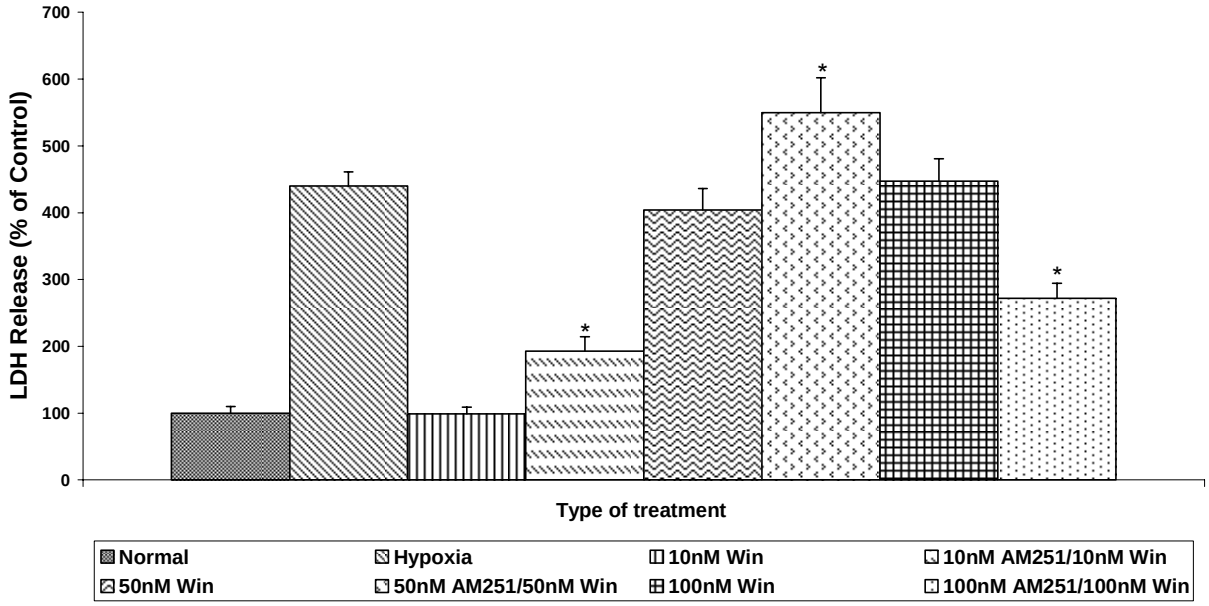
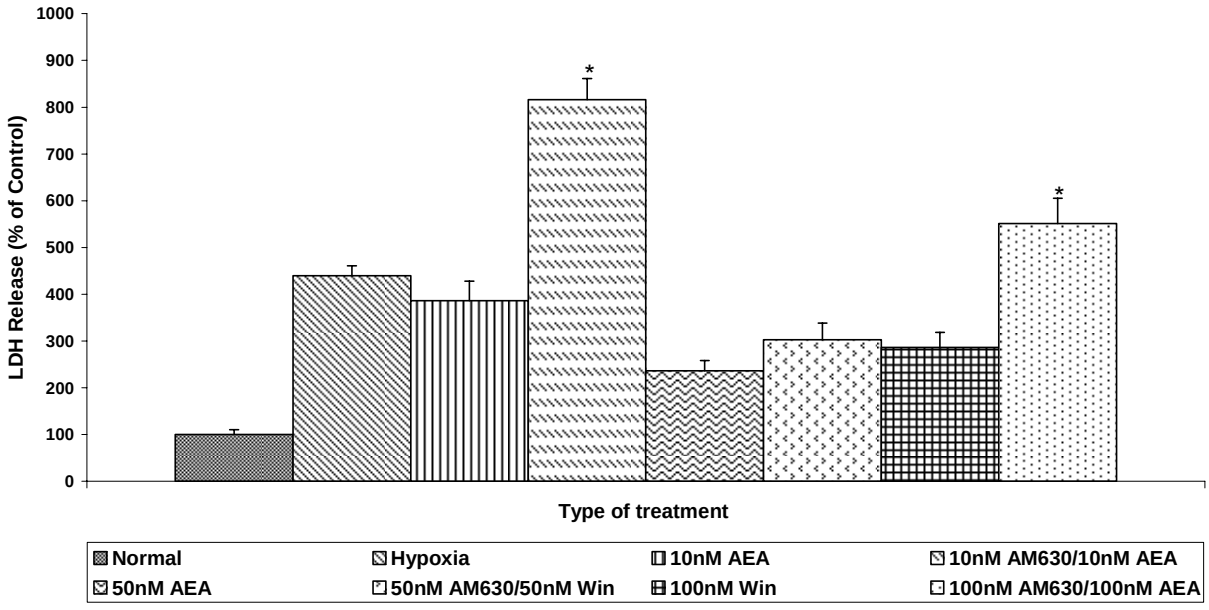


Fig.3.19b.The effect of cannabinoid agonist/antagonist pre-treatment on LDH release in cultured B50 cells in hypoxia



Figs.3.19a & b:The effect of cannabinoid agonist pre-treatment in the presence or absence of antagonist against hypoxia on LDH release in B50 cells cultured in hypoxia ($5\%O_2;5\%CO_2$), using LDH assay. Cells were concurrently cultured and treated with the agonists only or agonist/antagonists at 0hr and then cultured for a total of 96hrs. The absorbance (n=6) was measured at 490nm. The LDH released from the normal untreated culture (21% O_2; 5% CO_2) was used as the control (100%) and results expressed relative to the control. (*$P<0.05$ versus agonist pre-treated hypoxic cells; Student's t-test).

Table 3.13: The effect of cannabinoid pre-treatment on LDH release on cultured B50 cell in hypoxia

Type of Treatment	Measured Optical Density(OD)	Percent of Control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	0.911±0.211	440±21.1*
10nM Win	0.206±0.101	99±10.1$^{\#}$
50nM Win	0.836±0.321	404±32.1*
100nM Win	0.926±0.335	447±33.5*
10nM AEA.	0.799±0.422	386±42.2*
50nM AEA.	0.488±0.225	236±22.5*$^{\#}$
100nM AEA.	0.590±0.321	285±32.1*$^{\#}$
10nM 2-AG.	1.400±0.456	677±45.6*$^{\#}$
50nM 2-AG.	0.428±0.115	206±11.5*$^{\#}$
100nM 2-AG.	1.021±0.441	494±44.1*

The effect of cannabinoid agonist pre-treatment on B50 cells concurrently exposed to hypoxia (5%O_2; 5%CO_2) and cannabinoid agonist on LDH release, using LDH assay. Cells were pre-treated at 0hr and then cultured for 96hrs in hypoxia and then assayed for LDH release. The absorbance (n=6) was measured at 490nm. The LDH released from the normal untreated culture (21%O_2; 5%CO_2), was used as the control (100%) and the result was expressed relative to the control. (Data expressed as mean ±SD; *$P<0.05$ versus normal; $^{\#}P<0.05$ versus hypoxic untreated cells; Student's t-test).

Table 3.14: The effect of cannabinoid agonist/antagonist pre-treatment on LDH release in cultured B50 cells in hypoxia.

Type of Treatment	Measured Optical Density(OD)	Percent of Control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	0.911±0.211	440±21.1
10nM Win	0.206±0.101	99±10.1
10nM AM251/ 10nM Win	0.400±0.212	193±10.1*
50nM Win	0.836±0.321	404±32.1
50nM AM251/ 50nM Win	1.380±0.522	550±52.2*
100nM Win	0.926±0.335	447±33.5
100nM AM251/ 100nM Win	0.563±0.221	272±22.1*
10nM AEA	0.799±0.422	386±42.2
10nM AM630/ 10nM AEA	1.688±0.455	816±45.5*
50nM AEA	0.488±0.225	236±22.5
50nM AM630/ 50nM AEA	0.627±0.352	303±35.2
100nM AEA	0.590±0.321	285±32.1
100nM AM630/ 100nM AEA	1.140±0.542	551±54.2*

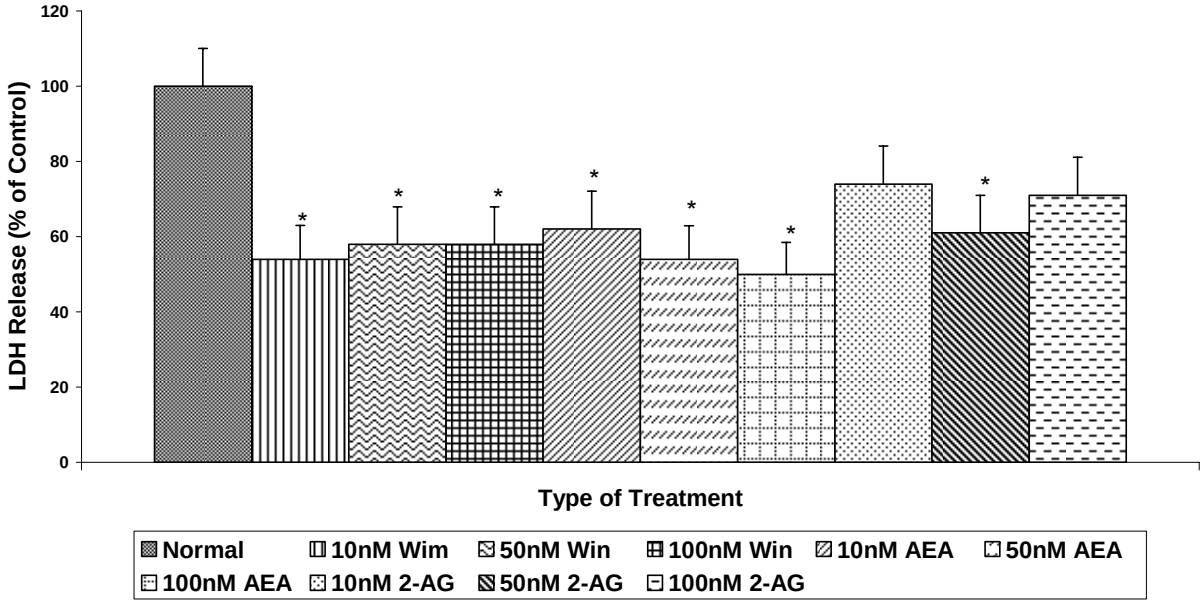
The effect of cannabinoid agonist in the presence of antagonist pre-treatment against hypoxia on LDH release in B50 cells cultured in hypoxia ($5\%O_2$;$5\%CO_2$), using LDH assay. Cells were cultured and pre-treated at 0hr and cultured for a total of 96hrs in hypoxia and assayed for LDH release. The absorbance (n=6) was measured at 490nm. The LDH released from the normal untreated culture ($21\%O_2$; $5\%CO_2$), was used as the control (100%) and the result was expressed relative to the control. (Data expressed as mean ±SD; *$P<0.05$ versus agonist/antagonist treated cells; Student's t-test).

Table 3.15: The effect of cannabinoid pre-treatment on LDH release in normal cultured B50 cells.

Type of Treatment	Measured Optical Density(OD)	Percent of Control (% of Control)
Normal no Drug	0.172±0.122	100±12.2
10nM Win	0.092±0.090	54±9.0*
50nM Win	0.100±0.099	58±9.9*
100nM Win	0.100±0.099	58±9.9*
10nM AEA	0.106±0.101	62±10.1*
50nM AEA	0.092±0.089	54±8.9*
100nM AEA	0.086±0.085	50±8.5*
10nM 2-AG	0.126±0.101	74±10.1
50nM 2-AG	0.104±0.100	61±10.0*
100nM 2-AG	0.122±0.101	71±10.1

The effect of cannabinoid agonist concurrent treatment and culture of B50 cells on LDH release in normal cultured B50 cells (21%O_2; 5%CO_2), using LDH assay. Cells were pre-treated with cannabinoid agonist at 0hr and then cultured for a total of 96hrs in normal culture and the absorbance (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells was used as the control (100%) and LDH release was expressed relative to the control. (Data presented as mean ±SD; *$P<0.05$ versus untreated normal cells; Student's t-test).

Fig.3.20.The effect of cannabinoid pre-treatment on LDH release in normal B50 cells in culture



The effect of cannabinoid agonist pre-treatment on LDH release in normal cultured B50 cells ($21\%O_2;5\%CO_2$), using LDH assay. Cells were treated with cannabinoid agonists at 0hr of culture and then cultured for a total of 96hrs in normal conditions. The LDH assay absorbance (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells was used as the control (100%) and LDH release was expressed relative to the control. (*$P<0.05$ versus untreated normal cells; Student's t-test).

3.5 The effect of DbcAMP on LDH release from cultured B50 cells

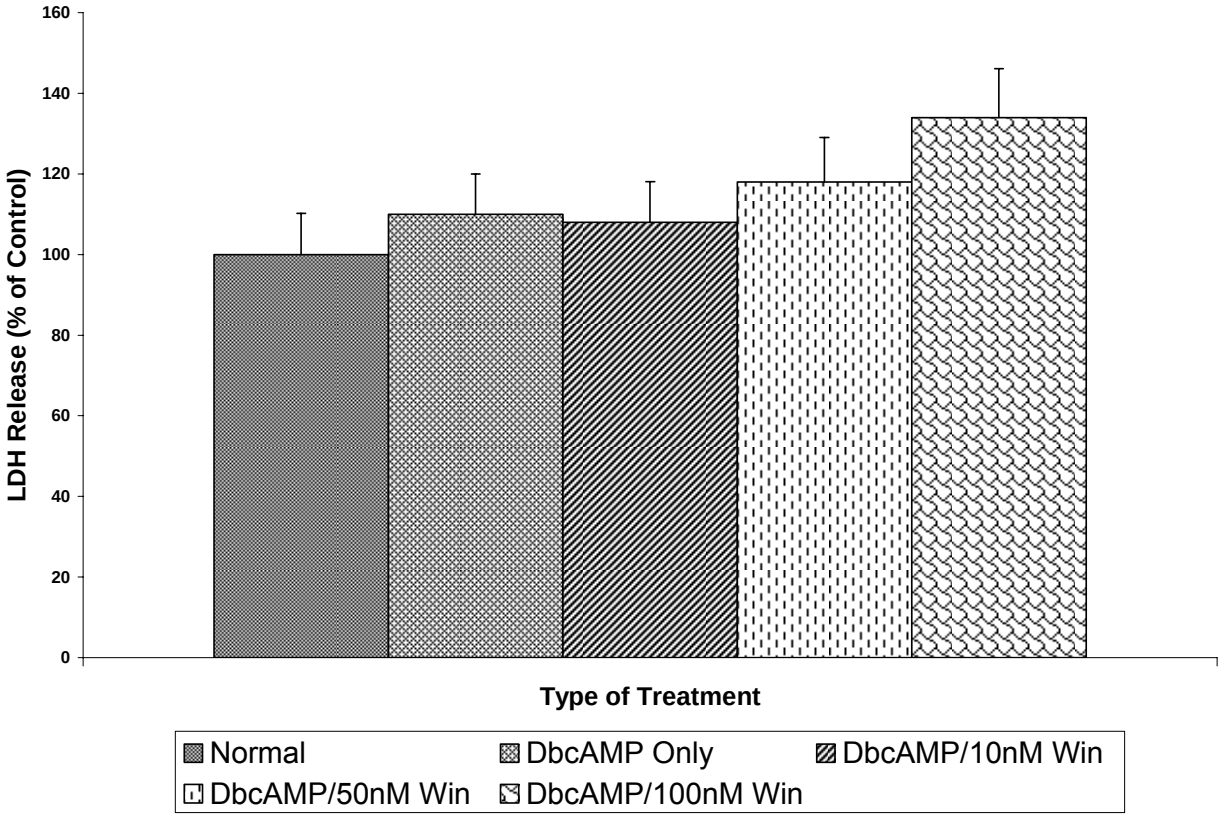
3.5.1 Effect of DbcAMP and cannabinoid agonists treatment ± antagonist on LDH release in cultured normal B50 cells.

The effect of 1mM dibutyl cyclic adenosine monophosphate (DbcAMP) administration and cannabinoid agonist treatment on LDH release in normal cultured B50 cells, show a non-significant increase in LDH release from the cells treated with different concentrations of cannabinoid agonist and 1mM concentration of DbcAMP when compared with the normalized LDH release from the untreated normal cultured cells (100%). The results as shown in Figure 3.21, are normal cells with only DbcAMP (110%), DbcAMP with 10nM Win (108%), DbcAMP with 50nM Win (118%) and DbcAMP with 100nM Win (134%). When the result of LDH release from B50 cells treated with DbcAMP only (110%) was compared to those treated with DbcAMP and different concentrations of cannabinoid (Win) agonist, the result show a non-significant increase from DbcAMP with 50nM Win (118%) and DbcAMP with 100nM Win (134%), and a non-significant decrease in LDH release from DbcAMP with 10nM Win (108%).

The effect of normal culture of B50 cells with 1mM DbcAMP, pre-treated with different concentrations of cannabinoid agonists is shown in Figure 3.22. The results show an increase in LDH release from cannabinoid agonist pre-treated and DbcAMP administered cells in normal culture when compared to the untreated normal cultured B50 cells (100%). The results are cells with only DbcAMP (110%), DbcAMP with 10nM Win (109%), DbcAMP with 50nM Win (175%) and DbcAMP with 100nM Win (188%). The increase in LDH leakage was significant in those cells pre-treated with DbcAMP and 50nM Win (175%); and DbcAMP with 100nM Win (188%) ($P<0.05$), while the increase in LDH release in those B50 cells treated with DbcAMP only (110%) ($P=0.98$), DbcAMP with 10nM Win (109%) ($P=0.77$), was not significant. When the LDH release in B50 cells pre-treated with DbcAMP only (110%) was compared with those pre-treated with DbcAMP and cannabinoid agonist, the result showed a pattern of significance similar to that seen when compared with the normal untreated B50 cells.

The result of the culture of B50 cells in 1mM DbcAMP and treated with cannabinoid agonists in the presence of antagonists on LDH release in normal cultured B50 cells is shown in Figure 3.23. The results show that the LDH release was significantly increased ($P<0.05$) in those treated with 50nM AM251/50nM Win (130%) and 100nM AM251/100nM Win (191%) when compared with untreated normal B50 cells (100%). When the LDH release was compared between the cells treated with DbcAMP only (110%) and the other groups, the result showed a significant increase ($P<0.05$) in LDH release in those B50 cells treated with 100nM AM251/100nM Win (191%), a non-significant increase in 50nM AM251/50nM Win (130%) ($P=0.11$) and a non-significant decrease in 10nM AM251/10nM Win (107%) ($P=0.76$).

Fig.3.21.The effect of cannabinoid/ DbcAMP treatment on LDH release in normal cultured B50 cells.



The effect of cannabinoid agonist/DbcAMP treatment on LDH release in normal cultured B50 cells ($21\%O_2$; $5\%CO_2$) using LDH assay. Cells were cultured in the presence of 1mM concentration of DbcAMP for 48hrs and then treated with different concentrations of cannabinoid agonist (Win) for 48hrs for a total of 96hrs of normal culture and the LDH assay absorbance (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells, was normalized and used as the control (100%) and LDH release was expressed relative to the control.(Data as Mean ±SD; Student's t-test).

Fig.3.22.The effect of Cannabinoid/DbcAMP pre-treatment on LDH release in normal B50 cells.

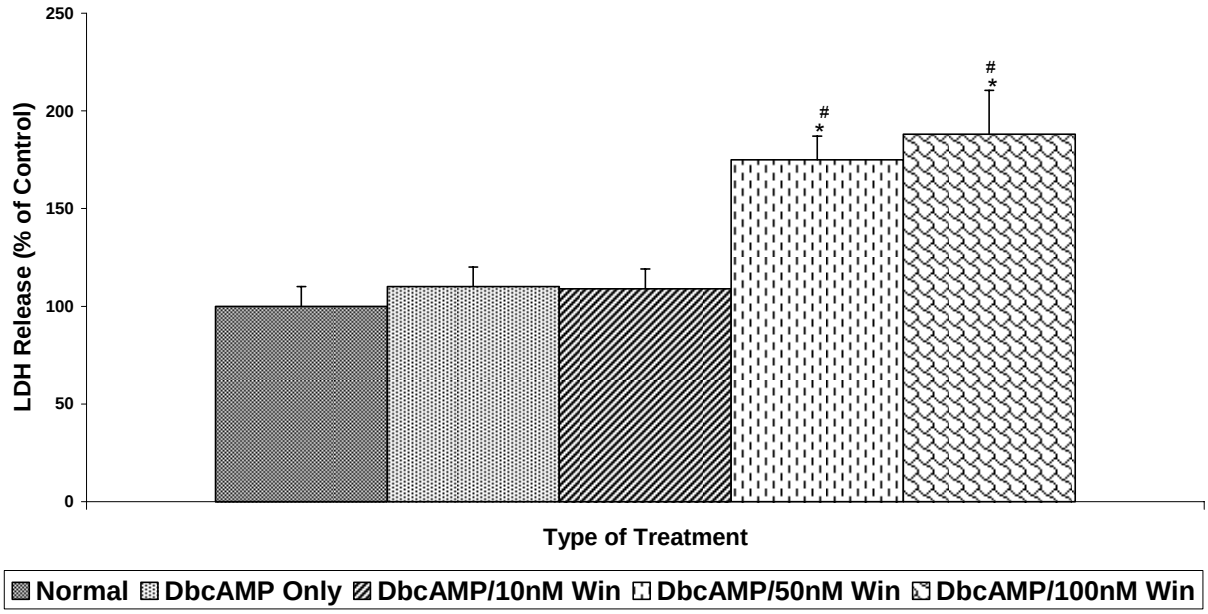
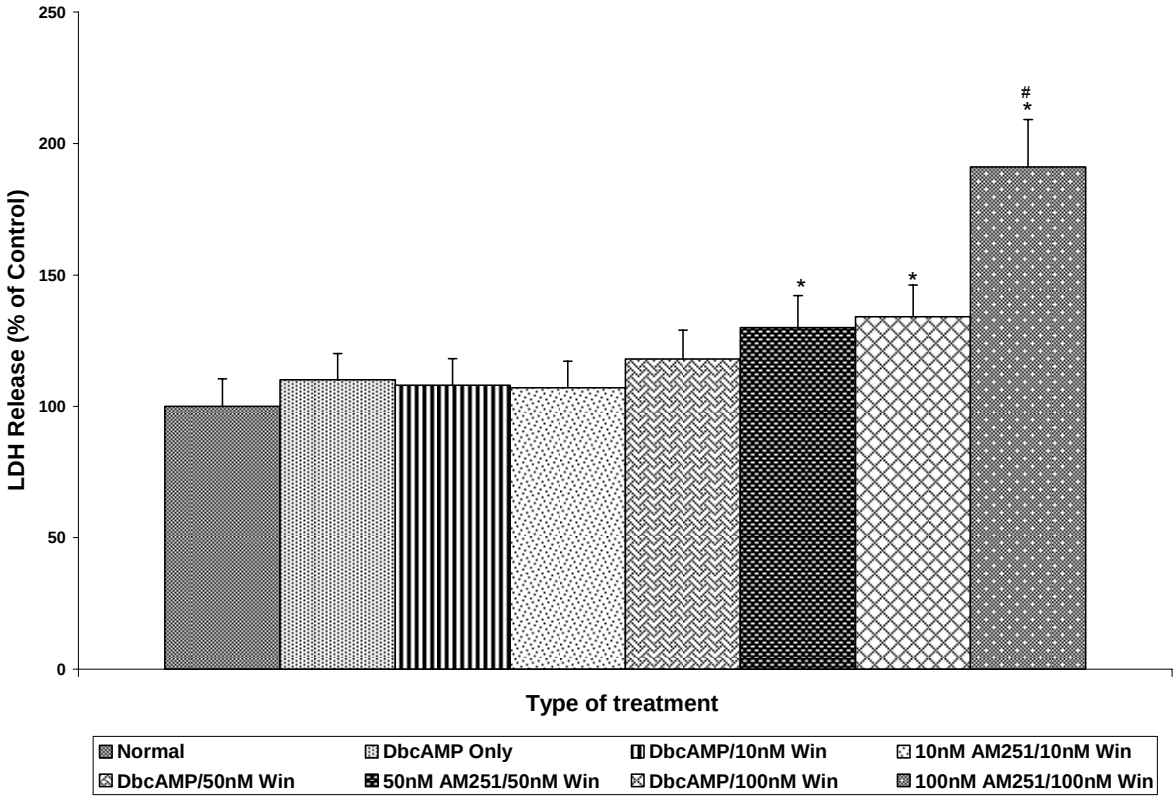


Fig.3.22: Effect of cannabinoid agonist/DbcAMP pre-treatment on LDH release in normal cultured B50 cells (21%O_2; 5%CO_2), using LDH assay. Cells were cultured in 1mM DbcAMP and pre-treated with cannabinoid agonist at 0hr and then cultured for 96hrs in normal culture and the absorbance (n= 6) was measured at 490nm. The LDH released from the untreated normal cultured cells, was normalized and used as the control (100%) for the experiment and LDH release was expressed relative to the control. (Data point as mean ±SD; *$P<0.05$ versus untreated normal cells in culture; $^{\#}P<0.05$ versus cells with DbcAMP only; Student's t-test).

Fig.3.23. The effect of DbcAMP and cannabinoid agonist/antagonist treatment on LDH release in normal B50 cells



The effect of DbcAMP with cannabinoid agonist/antagonist treatment on LDH release in normal cultured B50 cells (21%O_2; 5%CO_2), using LDH assay. Cells were cultured in the presence of 1mM concentration of DbcAMP for 48hrs and then treated with different concentrations of cannabinoid agonist (Win) and antagonist AM251 for 48hrs making up a total of 96hrs of normal culture. The absorbance of the LDH assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells, was normalized and used as the control (100%) and LDH release was expressed relative to the control.(Data as mean ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus Cells in DbcAMP only; Student's t-test).

3.5.2 Effect of DbcAMP and cannabinoid agonists treatment and pre-treatment ± antagonist on LDH release in cultured B50 cells in hypoxia.

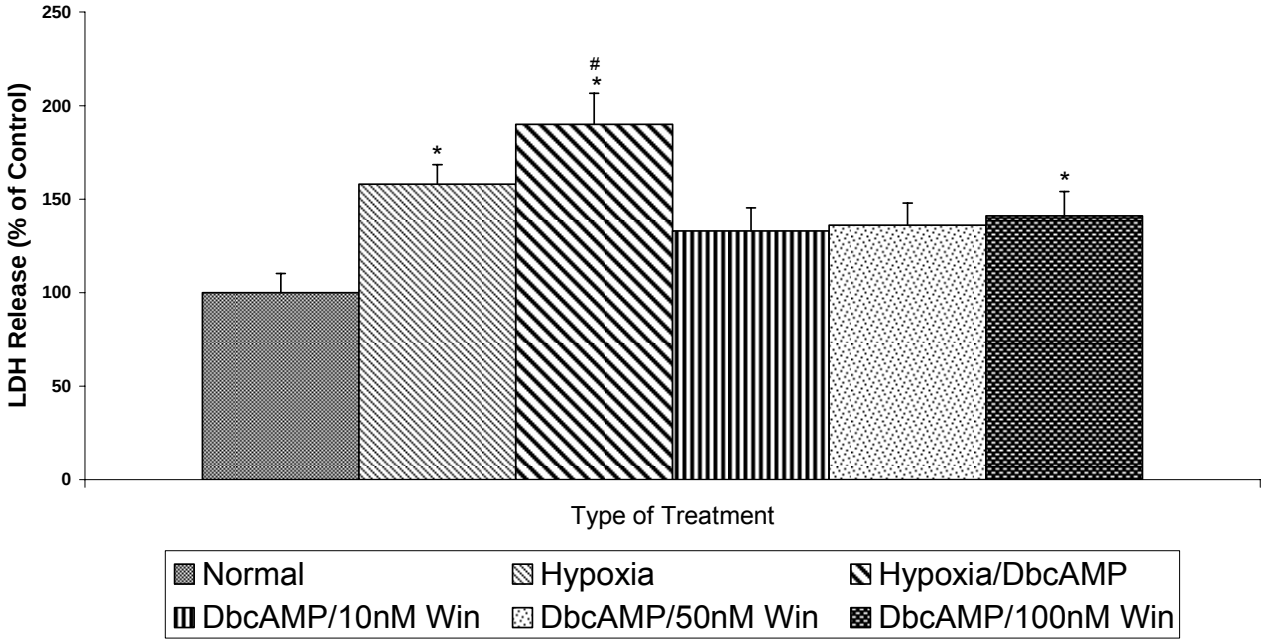
The effect of B50 cell culture in 1mM DbcAMP, treated with different concentrations of cannabinoid agonist on LDH release in hypoxia is shown in Figure 3.24. The results show a significant ($P<0.05$) increase in LDH release from untreated B50 cells in hypoxia (158%), hypoxia with DbcAMP only (190%), and DbcAMP with 100nM Win (141%). There was a non-significant increase in DbcAMP with 10nM Win (133%) ($P=0.91$) and DbcAMP with 50nM Win (136%) ($P=0.87$). When the LDH release from the hypoxic untreated B50 cells (158%) was compared with cells treated with different concentrations of DbcAMP and cannabinoid agonist, the result show a significant increase ($p<0.05$) in LDH release in hypoxia with DbcAMP only (190%); a non-significant increase from DbcAMP with 100nM Win (141%) ($P=0.22$), and a non-significant decrease in LDH release from DbcAMP with 10nM Win (133%) ($P=0.15$) and DbcAMP with 50nM Win (136%) ($P=0.23$).

The effect of B50 cell culture in 1mM DbcAMP, pretreated against hypoxia using different concentrations of cannabinoid agonist on LDH release is shown in Figure 3.25. There was a significant increase ($P<0.05$) in LDH leakage from cells in different pre-treatment groups when compared with the untreated normal cells. The LDH release in the different groups are, hypoxia with no drug (158%), hypoxia with only DbcAMP (190%), DbcAMP with 10nM Win (146%), DbcAMP with 50nM Win (183%) and DbcAMP with 100nM Win (191%). When the level of LDH release was compared between the untreated hypoxic B50 cells (158%) and treated hypoxic cells, the results show that LDH release was significantly increased ($P<0.05$) in hypoxia with DbcAMP only (190%), DbcAMP with 50nM Win (183%) and DbcAMP with 100nM Win (191%), while there was a non-significant decrease when compared to DbcAMP with 10nM Win (146%) ($P=0.58$).

The culture of B50 cells in 1mM DbcAMP and treated with cannabinoid agonists in the presence of the antagonists on LDH release in B50 cells in hypoxia is shown in Figure 3.26. The results show that the LDH release from cells cultured with different concentrations of cannabinoid agonist/antagonist in hypoxia were significantly increased ($P<0.05$) in cells treated with 10nM AM251/10nM Win (182%) and

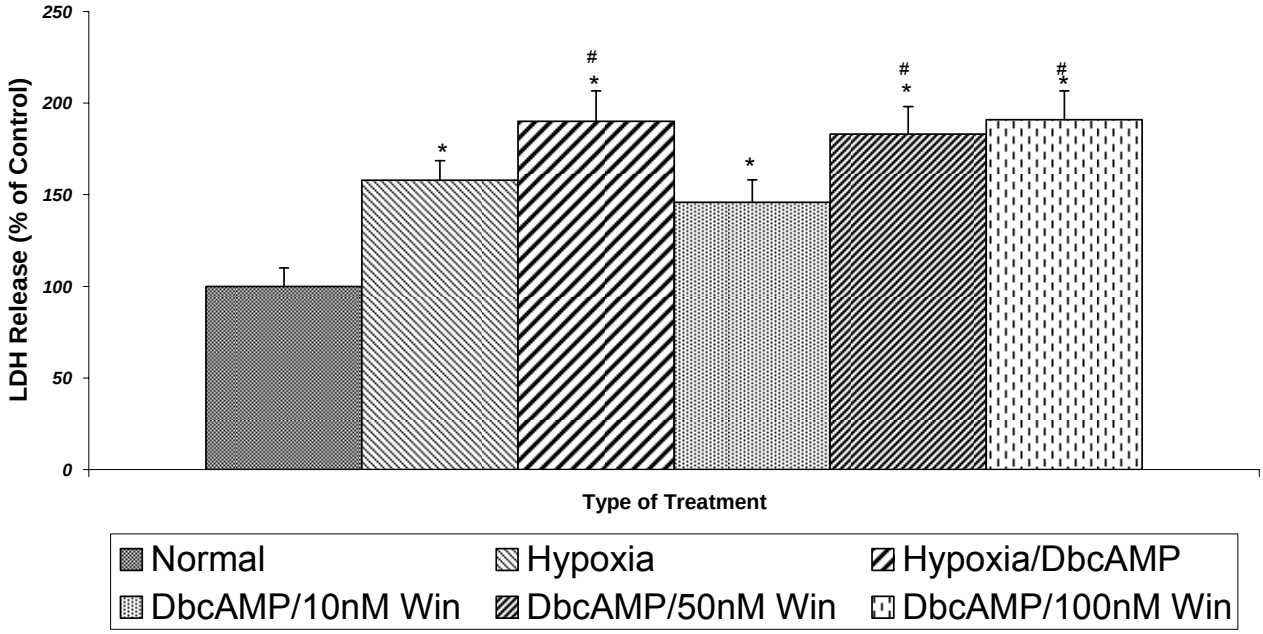
AM251/100nM Win (213%), when compared to the cells treated with agonist only DbcAMP/10nM Win (146%) and DbcAMP/100nM Win (191%).

Fig.3.24.The effect of cannabinoid agonist/DbcAMP treatment on LDH release on B50 cells in Hypoxia



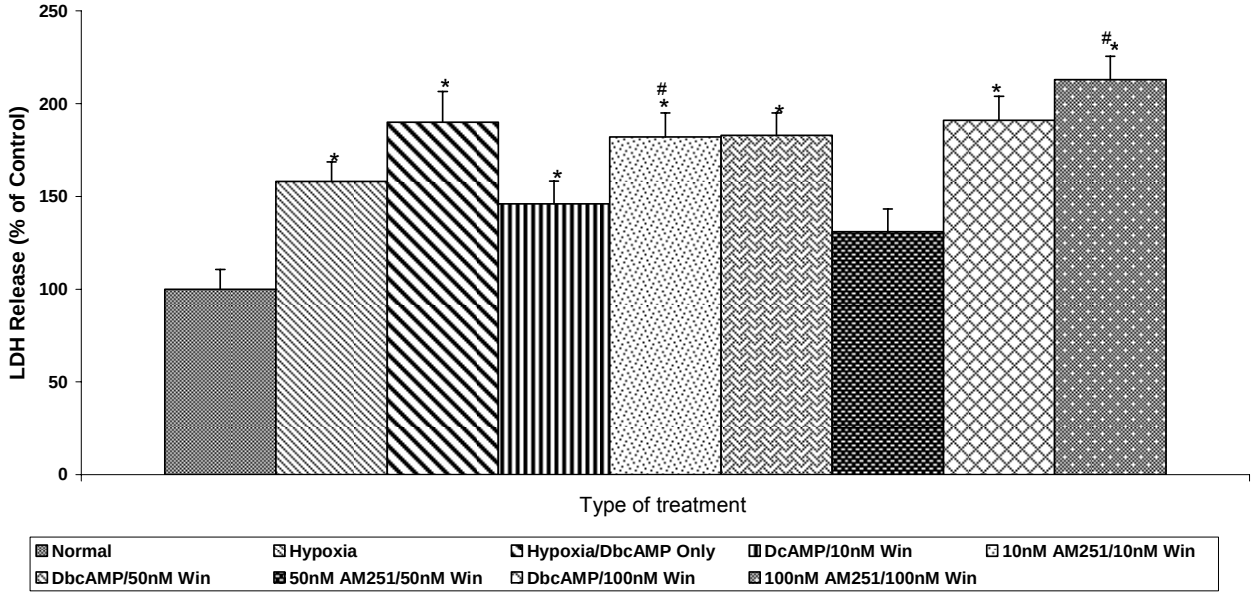
The effect of cannabinoid agonist and DbcAMP treatment on LDH release in B50 cells cultured in hypoxia ($5\%O_2;5\%CO_2$), using LDH assay. Cells were cultured in the presence of 1mM concentration of DbcAMP for 48hrs and treated with different concentrations of cannabinoid agonist (Win) for 48hrs for a total of 96hrs of hypoxic culture. The LDH was assayed and absorbance (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells ($21\%O_2$; $5\%CO_2$), was normalized and used as the control (100%) and LDH release was expressed relative to the control.(Data as Mean ±SD; *$P<0.05$ versus normal control; $^{\#}P<0.05$ versus untreated hypoxic cells; Student's t-test).

Fig.3.25.The effect of cannabinoid/DbcAmp pre-treatment on LDH release in B50 cells in Hypoxia.



The effect of cannabinoid agonist pre-treatment against hypoxia in the presence of DbcAMP on LDH release in B50 cells cultured in hypoxia ($5\%O_2;5\%CO_2$), using LDH assay. Cells were cultured in 1mM DbcAMP and pre-treated against hypoxia with cannabinoid agonist at 0hr and then cultured for 96hrs in culture and the LDH assay absorbance (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells ($21\%O_2$; $5\%CO_2$), was normalized and used as the control (100%) and LDH release was expressed relative to the control. (Data as Mean ±SD *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus untreated hypoxic cells; Student's t-test).

Fig.3.26. The effect of DbcAMP and cannabinoid agonist/antagonist treatment on LDH release in B50 cells in hypoxia



The effect of B50 cell culture in DbcAMP and treated with cannabinoid agonist in the presence or absence of the antagonist on LDH release in cultured B50 cells in hypoxia ($5\%O_2$; $5\%CO_2$), using LDH assay. Cells were cultured in the presence of 1mM concentration of DbcAMP for 48hrs and then treated with different concentrations of cannabinoid agonist (Win) and antagonist (AM251) for 48hrs for a total of 96hrs of hypoxic culture. The absorbance of the LDH assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells ($21\%O_2$; $5\%CO_2$), was normalized and used as the control (100%) and LDH release was expressed relative to the control.(Data as mean ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}$ $P<0.05$ versus agonist treated cells in hypoxia; Student's t-test).

3.6 The concentration-dependent effect of a CB_1 antagonist on cannabinoid agonist.

3.6.1 Concentration-dependent activity of a selective CB_1 antagonist (AM251) on 10nM Win agonist treatment on LDH release in cultured B50 cells in hypoxia.

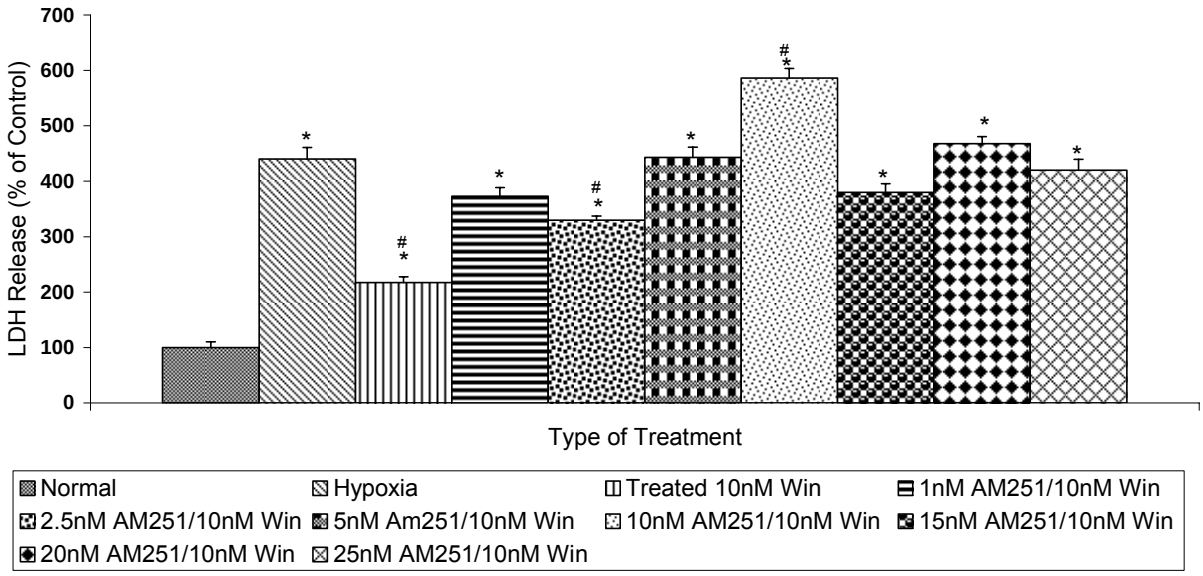
The effect of concentration on the activity of a selective cannabinoid antagonist (AM251) on cannabinoid agonist (10nM Win) treatment using LDH release as an indicator of cell damage in B50 cells cultured in hypoxia is shown in Figure 3.27. The results show different concentrations of the antagonist (AM251) used to reverse the effect of a fixed concentration of the agonist (10nM Win). The result of LDH leakage from B50 cells treated with the agonist (10nM Win) and the antagonist are; 1.0nM AM251 (373%); 2.5nM AM251 (330%), 5.0nM AM251 (443%), 10nM AM251 (586%), 15nM AM251 (380%), 20nM AM251 (468%) and 25nM AM251 (420%). The results show that treatment of B50 cells with 10nM Win and 5.0nM AM251 concurrently, brought the LDH release to 443% when compared to the agonist 10nM Win alone (217%) and untreated hypoxic B50 cells (440%). This shows that 5.0nM AM251 could be an ideal concentration of the antagonist to abolish the activity of the agonist (10nM Win) in the treatment of B50 cells in hypoxia. The result showed a significant increase ($P<0.05$) in LDH release in the experimental groups when compared to the normal cells. However, when the LDH release from the B50 cells in hypoxia (440%) was compared with those treated with different concentrations of the antagonist, the result showed a significant increase ($P<0.05$) in LDH release in 10nM AM251 (586%), while there was a significant decrease ($p<0.05$) in LDH release from hypoxic cells with 10nM Win treatment only (217%) and 2.5nM AM251 (330%) (Table 3.16).

Table 3.16: Concentration-dependent activity of a selective CB_1 antagonist (AM251) on 10nM Win agonist treatment on LDH release in cultured B50 cells in hypoxia

Type of Treatment	Measured Optical Density(OD)	Percent of Control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	0.911±0.211	440±21.1*
10nM Win	0.450±0.102	217±10.2*#
1.0nM AM251	0.772±0.153	373±15.3*
2.5nM AM251	0.684±0.074	330±7.4*#
5.0nM AM251	0.886±0.182	443±18.2*
10nM AM251	1.215±0.173	586±17.3*#
15nM AM251	0.771±0.155	380±15.5*
20nM AM251	0.969±0.123	468±12.3*
25nM AM251	0.870±0.192	420±19.2*

The effect of concentration-dependent activity of a selective CB_1 antagonist on the agonist in B50 cells cultured in hypoxia. B50 cells were cultured in hypoxia ($5\%O_2$; $5\%CO_2$) and treated with different concentrations of the cannabinoid antagonist (AM251) against a fixed concentration of the agonist (10nM Win) using LDH assay method. Cells were cultured for 48hrs and then treated with cannabinoid agonist (10nM Win) and different concentrations of the cannabinoid antagonist (AM251) and then cultured for 48hrs for a total of 96hrs of culture. The absorbance of LDH assay (n=6) was measured at 490nm and the LDH released from the untreated normal cultured cells ($21\%O_2$; $5\%CO_2$), was normalized and used as the control (100%) and the level of LDH release was expressed relative to the control. (Data presented as mean ±SD; *$P<0.05$ versus untreated normal cells; #$P<0.05$ versus hypoxia untreated cells; Student's t-test).

Fig.3.27. Concentration dependent activity of the antagonist (AM251) on agonist (10nM) Win treatment on LDH release in B50 cells in hypoxia



The effect of concentration-dependent activity of a selective CB_1 antagonist (AM251) on the agonist (10nM Win) in B50 cells cultured in hypoxia ($5\%O_2$; $5\%CO_2$), using different concentrations of the cannabinoid antagonist against a fixed concentration of the agonist (10nM Win) treatment on LDH assay. Cells were cultured for 48hrs and then treated with the cannabinoid agonist (10nM Win) and different concentrations of the antagonist (AM251) and then cultured for another 48hrs making a total of 96hrs of culture. The LDH assay absorbance (n=6) was measured at 490nm and the LDH released from the untreated normal cultured cells ($21\%O_2$; $5\%CO_2$), was normalized and used as the control (100%) and the level of LDH release was expressed relative to the control. (Data presented as mean ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus hypoxia, untreated cells; Student's t-test).

3.6.2 Concentration-dependent activity of a selective CB_1 antagonist (AM251) on 10nM Win pre-treatment using LDH release in cultured B50 cells in hypoxia.

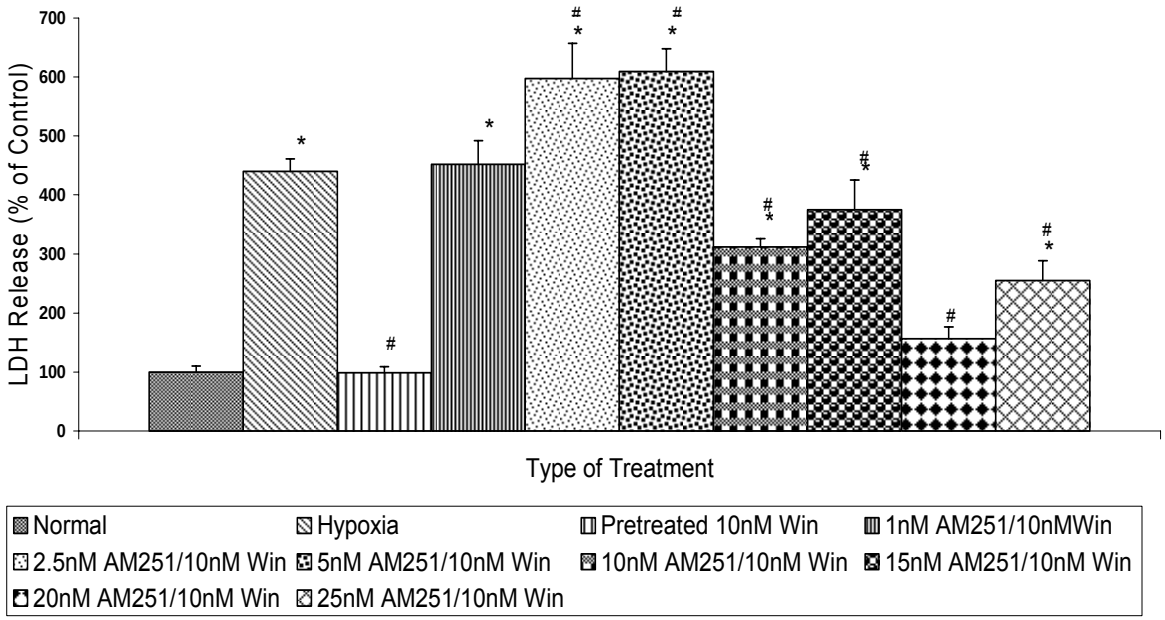
The effect of concentration-dependent activity of a selective CB_1 antagonist (AM251) pre-treated against hypoxia with a fixed concentration of 10nM Win on LDH release in B50 cells in hypoxia is shown in Figure 3.28. The results show that normal cells had 100% LDH release, the untreated hypoxic cells with 440% and the cells in hypoxia pre-treated with 10nM Win only (99%), while the pre-treated cells with different concentrations of the antagonist on a fix concentration of the agonist (10nM Win), had a significant increase in LDH release between 2-to 5-fold when compared with the normal cells ($P<0.05$) as follows: 1.0nM AM251 (450%); 2.5nM AM251 (597%); 5.0nM AM251 (609%);10nM AM251 (312%); 15nM AM251 (375%); 20nM AM251 (156%); 25nM AM251 (255%). The result showed that the pre-treatment of B50 cells concurrently with 10nM Win and 1.0nM AM251 (452%) was able to abolish the effect of the agonist and brought LDH release close to the hypoxic level (440%). When the LDH release from the untreated hypoxic cells (440%) was compared with those pre-treated with 10nM Win agonist and different concentrations of the antagonist, the result showed a significant increase ($P<0.05$) in LDH release in those cells pre-treated with 2.5nM AM251 (597%) and 5.0nM AM251 (609%), and a significant decrease ($P<0.05$) in LDH release from those B50 cells pre-treated with 10nM AM251 (312%), 15nM AM251 (375%), 20nM AM251 (156%) and 25nM AM251 (255%) (Table 3.17).

Table 3.17: Concentration-dependent activity of a selective CB_1 antagonist (AM251) on 10nM Win pre-treatment using LDH release in cultured B50 cells in hypoxia

Type of Treatment	Measured Optical Density(OD)	Percent of Control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	0.911±0.211	440±21.1*
10nM Win	0.206±0.101	99±10.1$^{\#}$
1.0nM AM251	0.936±0.40	452±40.0*
2.5nM AM251	1.235±0.60	597±60.0*$^{\#}$
5.0µM AM251	1.260±0.39	609±39.0*$^{\#}$
10nM AM251	0.647±0.142	312±14.2*$^{\#}$
15nM AM251	0.777±0.50	375±50.0*$^{\#}$
20nM AM251	0.322±0.02	156±2.0$^{\#}$
25nM AM251	0.527±0.34	255±34.0*$^{\#}$

The effect of concentration-dependent activity of a selective CB_1 antagonist (AM251) on pre-treatment with the agonist (Win) in B50 cells cultured in hypoxia using LDH release. B50 cells were cultured in hypoxia ($5\%O_2$;$5\%CO_2$), using different concentrations of the selective cannabinoid (CB_1) antagonist (AM251) against a fixed concentration of cannabinoid agonist (10nM Win) using LDH assay. Cells were cultured and pre-treated against hypoxia with cannabinoid agonist (10nM Win) and with different concentrations of the antagonist at 0hrs and then cultured for a total of 96hrs. The absorbance of LDH assay (n=6) was measured at 490nm and the LDH released from the untreated normal cultured cells ($21\%O_2$; $5\%CO_2$), was normalized and used as the control (100%), the level of LDH release was expressed relative to the control. (Data expressed as mean ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus hypoxia untreated; Student's t-test).

Fig.3.28.Concentration dependent activity of antagonist (AM251) on the agonist (10nM Win) pre-treatment using LDH release in B50 cells in hypoxia.



The effect of concentration-dependent activity of the selective CB_1 antagonist (AM251) on the pre-treatment against hypoxia with the agonist (Win) in B50 cells cultured in hypoxia using LDH release. B50 cells were cultured in hypoxia ($5\%O_2$;$5\%CO_2$), using different concentrations of the cannabinoid antagonist (AM251) against a fixed concentration of the agonist (10nM Win). Cells were cultured and pre-treated with cannabinoid agonist (10nM Win) and different concentrations of the antagonist at 0hrs and then cultured for 96hrs. The absorbance of the LDH assay (n=6) was measured at 490nm and the LDH released from the untreated normal cultured cells ($21\%O_2$; $5\%CO_2$), was normalized and used as the control (100%) for the experiment, the level of LDH release was expressed relative to the control. (Data expressed as mean ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus hypoxia untreated; Student's t-test).

3.7 Opioid receptor agonist administration

3.7.1. The effect of opioid treatment on the morphology of cultured B50 Cells.

Morphological changes in untreated hypoxic B50 cells in culture are shown in Plates 23, 25 and 26 when compared to normal B50 cells in Plates 21, 22 and 24. The normal cells showed the anticipated morphology of the neuronal B50 cells with normal neuronal cell arrangements while the B50 cells in hypoxia, showed groups of dead and degenerating cells when compared with the normal cells in culture. The effect of opioid agonist treatment on B50 neuronal cells in hypoxia, showed some dead and degenerating B50 cells when compared with normal B50 cells but when compared with untreated hypoxic cells, the hypoxic opioid-treated B50 cells, showed reduced cell death and improved morphology of the neuronal cells, Plates 27-29 for different concentrations of opioid receptor agonist treatment.

The effect of pre-treatment against hypoxia with different concentrations of opioid agonists on the morphology of B50 cells in culture, show groups of degenerating cells as in Plates 30-35 for opioid pre-treated cells in hypoxia. The results show that the pre-treated B50 cells in hypoxia with different concentrations of opioid agonists, have improved morphology of the cells when compared with the untreated B50 cells in hypoxia. When the morphology of the pre-treated cells was compared to the B50 cells treated with the opioid agonist, results showed that the cells with opioid treatment had better and improved morphology of the cells. The results from the morphological changes in B50 cells either treated or pre-treated with opioid agonists, suggests that opioid agonists may have some therapeutic and protective benefits. When the results were further analyzed, they showed that the effect of hypoxia could have been aggravated by the length of time the cells stayed in hypoxia (Plates 23; 25 and 26), for 24, 72 and 96 hours of culture respectively. This is because the more cells are exposed to hypoxia, the more the damage to the cells resulting in more dead and degenerated cells in culture. The results also tend to suggest that these drug-induced changes may be concentration dependent in which there were more dead and degenerating cell aggregates at higher concentration of the opioid agonist especially with the cells pre-treated with the opioid agonists. This suggests that the B50 neuronal cells may show more sensitivity to the drugs when administered prior to exposure to hypoxic environment. Hence the higher the dose of the drugs, the more the adverse effects

especially when they are administered as a pre-treatment against hypoxia. Example is in the case of 100μM DAMGO administered as treatment (Plate 29) and pre-treatment (Plate 30), which showed that the treatment group was able to preserve the morphology of the B50 cells better than that of pre-treatment group in hypoxia. This could provide an explanation why some more adverse drug effects are experienced with some drugs in presymptomatic conditions rather than in symptomatic situations.

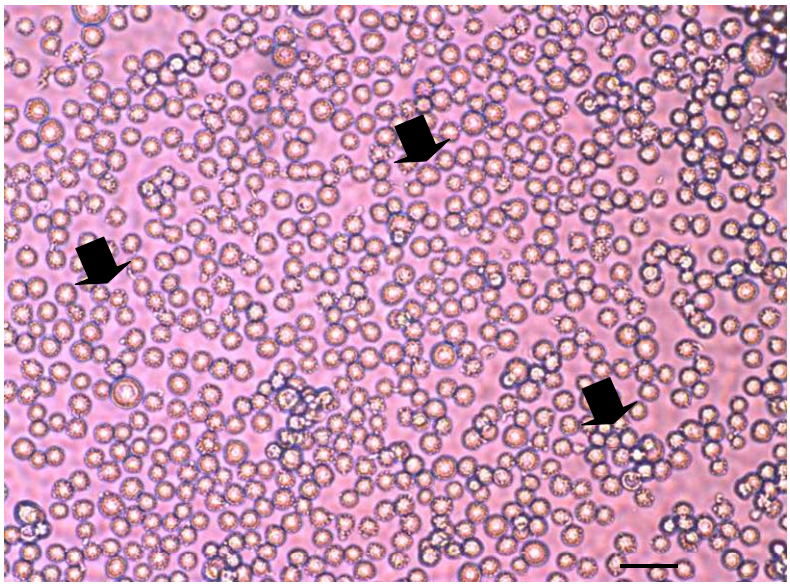


Plate 21:Representative of normal B50 cells at 0hrs of culture (21%O_2 and 5% CO_2) with normal cells (arrow). B50 cells was observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mmx40 magnification.

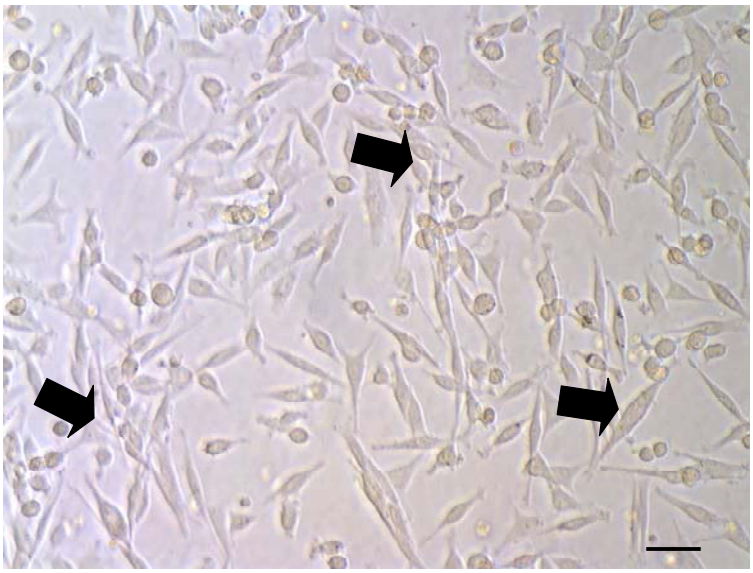


Plate 22:Representative of normal B50 cells at 24hrs of culture (21%O_2 and 5% CO_2) with normal cells (arrow). B50 cells was observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mmx40 magnification.

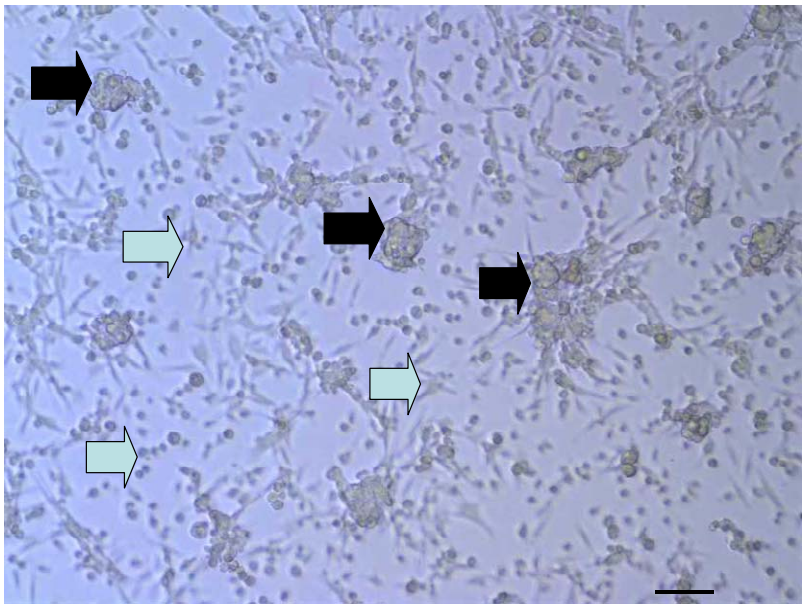


Plate 23:Representative of B50 cells in hypoxia at 24hrs of culture (5%O2 and 5% CO2) with some groups of degenerating cells (Black arrow) and some normal cells (Blue arrow). Cells was observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mmx20 magnification.

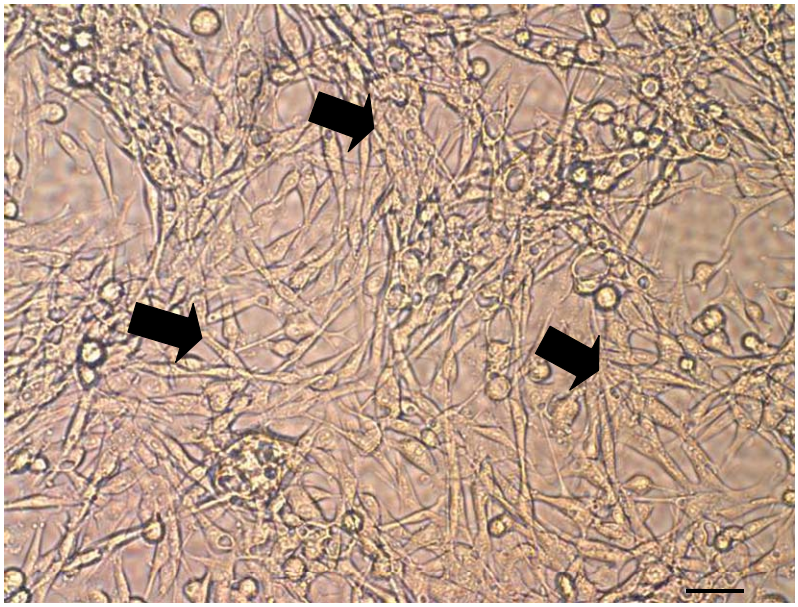


Plate 24:Representative of normal B50 cells at 72hrs of culture (21%O2 and 5% CO2) with normal cells (arrow). B50 cells was observed in six different plates with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mmx40 magnification.

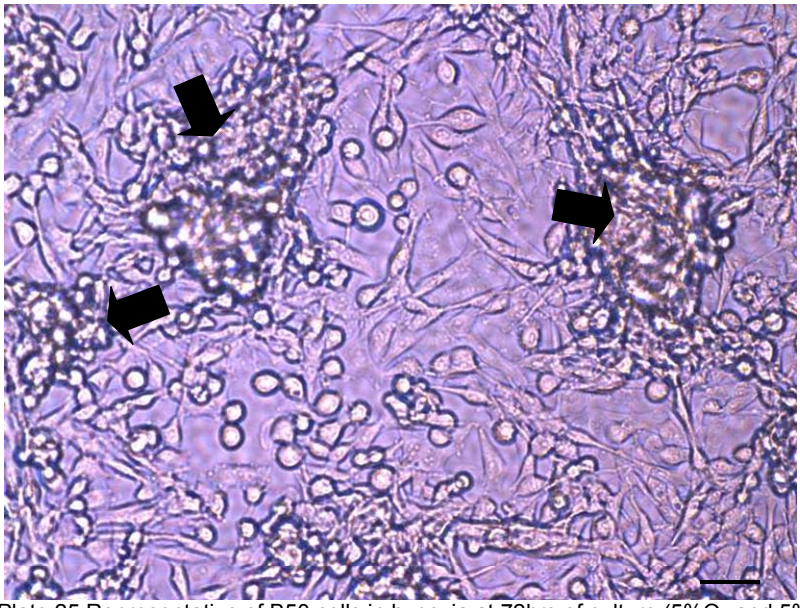


Plate 25:Representative of B50 cells in hypoxia at 72hrs of culture (5%O_2 and 5% CO_2) with groups of degenerating cells (arrow). B50 cells was observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mmx20 magnification.

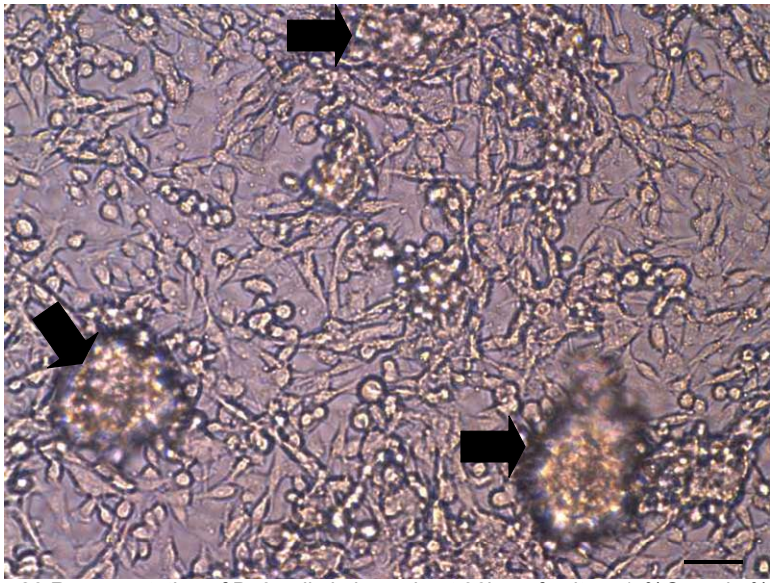


Plate 26:Representative of B50 cells in hypoxia at 96hrs of culture (5%O_2 and 5% CO_2) with groups of degenerating cells (arrow). B50 cells was observed in six different plates with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar =5mmx20 magnification.

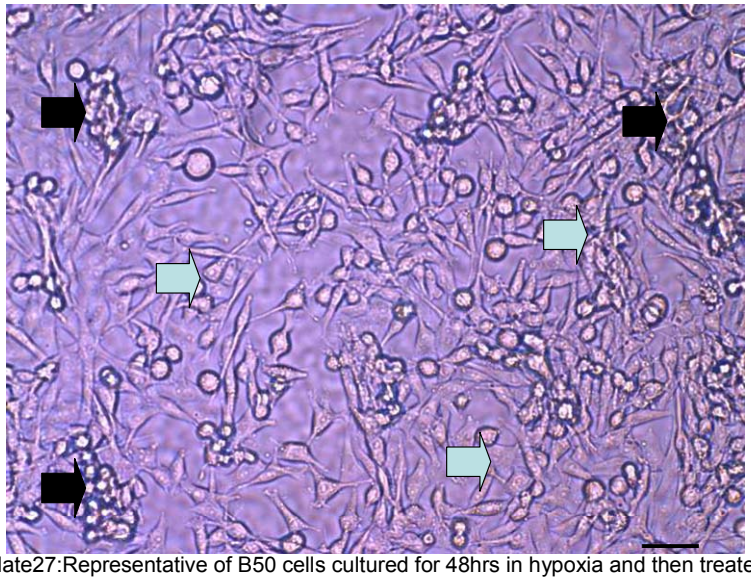


Plate27:Representative of B50 cells cultured for 48hrs in hypoxia and then treated with 10µM ICI-199,441 against hypoxia for 48hrs making-up 96hrs of culture (5%O_2 5% CO_2), showing many normal cells (Blue arrow) and few degenerating cells (Black arrow). The cell was observed in six different cultures with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

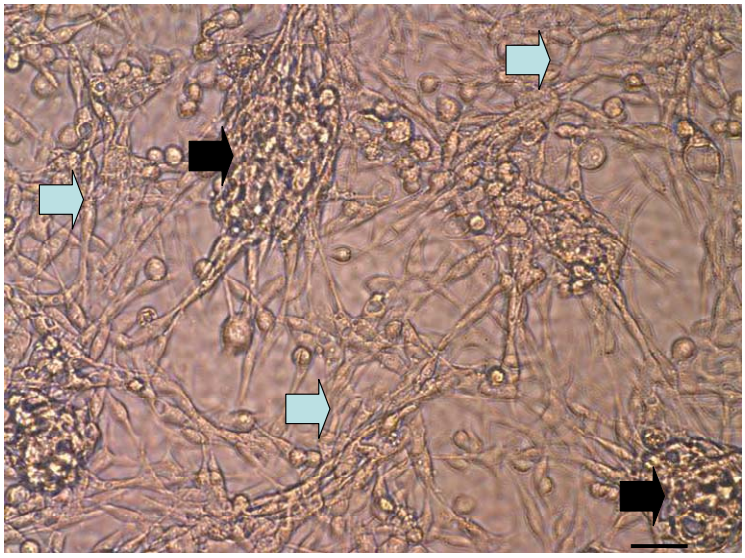


Plate28:Representative of B50 cells cultured for 48hrs in hypoxia and then treated with 50µM DSLET against hypoxia for 48hrs making-up 96hrs of culture (5%O_2 5% CO_2), showing many normal cells (Blue arrow) and few degenerating cells (Black arrow). The cell was observed in six different cultures with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

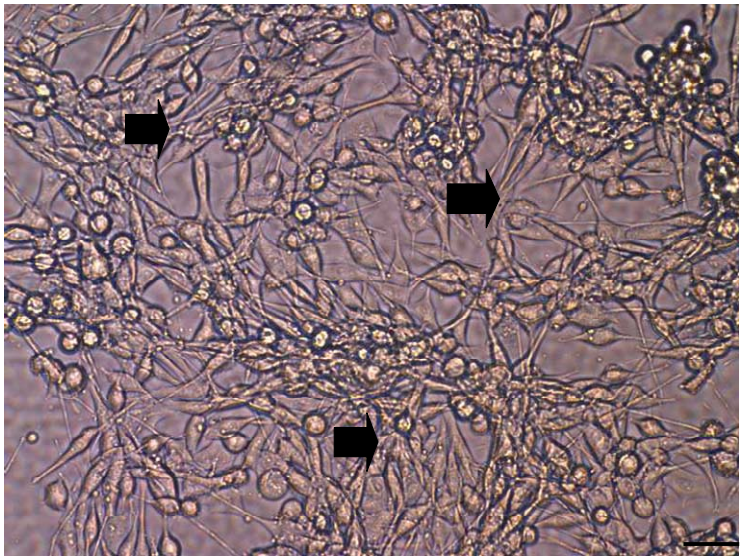


Plate29:Representative of B50 cells cultured for 48hrs in hypoxia and then treated with 100µM DAMGO against hypoxia for 48hrs making-up 96hrs of culture (5%O2 5% CO2), showing many normal cells (Black arrow). The cell was observed in six different cultures with same field morphological method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

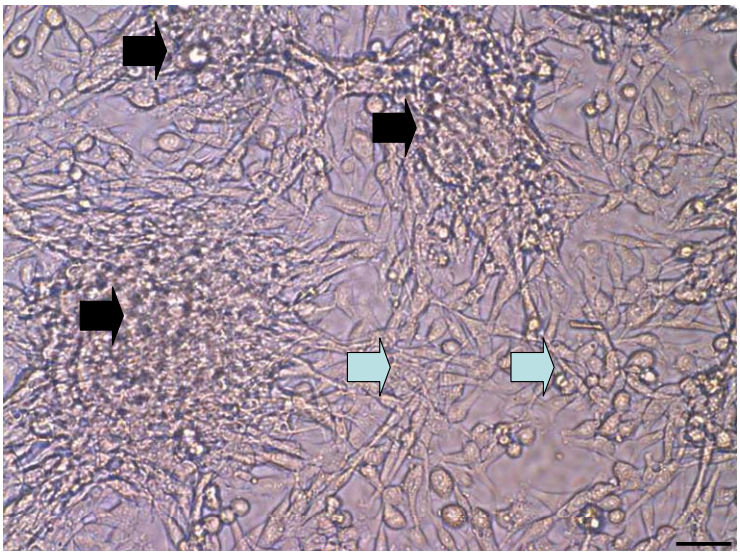


Plate30:Representative of B50 cells pre-treated with 100µM DAMGO against hypoxia for 96hrs of culture (5%O2 5% CO2), showing many degenerated cells (Black arrow) and some normal cells (Blue arrow). The cell was observed in six different cultures with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

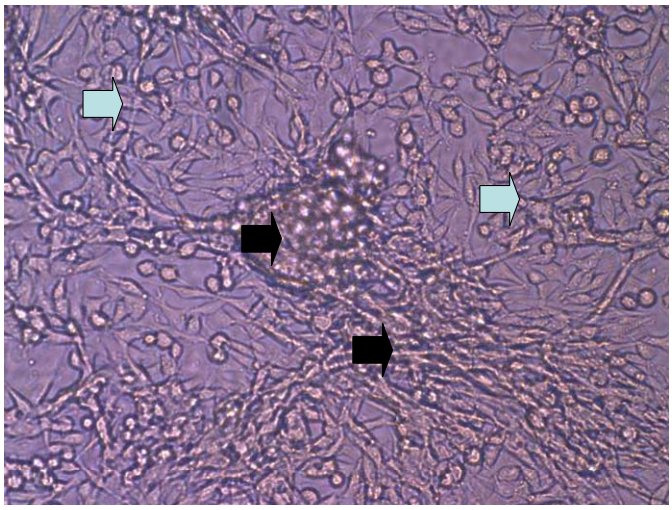


Plate31:Representative of B50 cells pre-treated with 100µM DSLET against hypoxia for 96hrs of culture (5%O2 5% CO2), showing few degenerated cells (Black arrow) and some normal cells (Blue arrow). The cells was observed in six different cultures with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

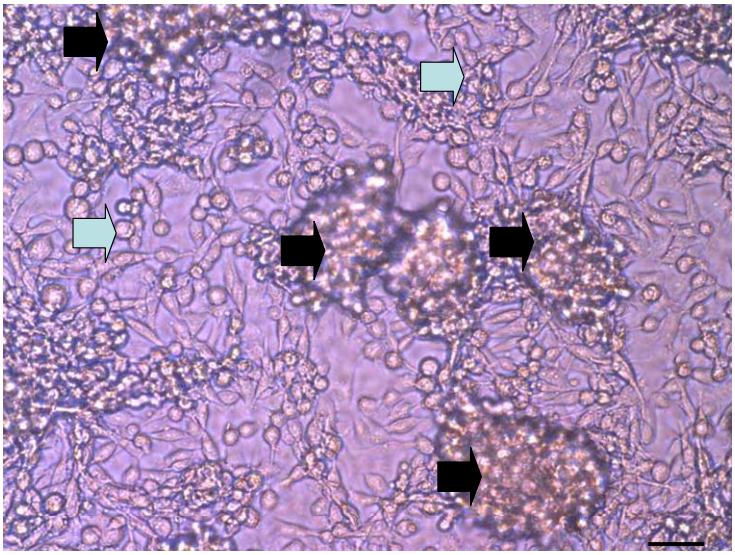


Plate32:Representative of B50 cells pre-treated with 100µM ICI-199,441 against hypoxia for 96hrs of culture (5%O2 5% CO2), with groups of degenerated cells (Black arrow) and some normal cells (Blue arrow). The cells was observed in six different cultures with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

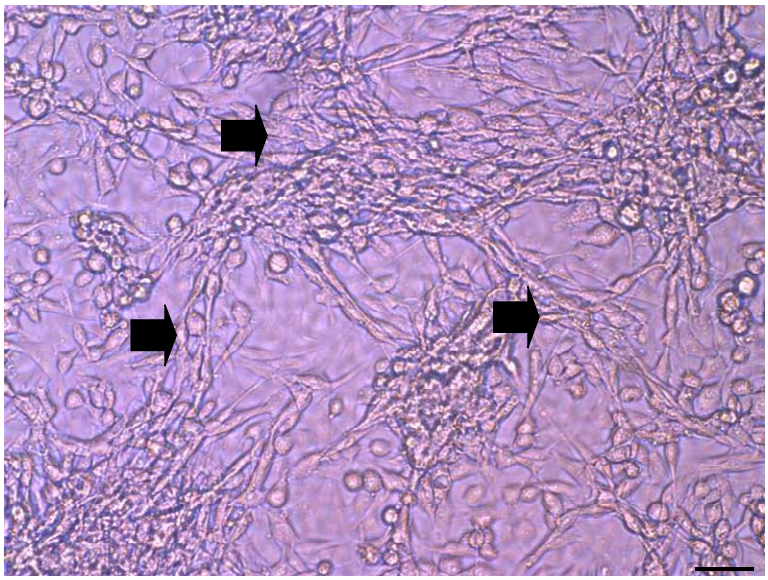


Plate33:Representative of B50 cells pre-treated with 10µM ICI-199,441 against hypoxia for 96hrs of culture (5%O2 5% CO2), with many normal cells (arrow). The cells was observed in six different cultures with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

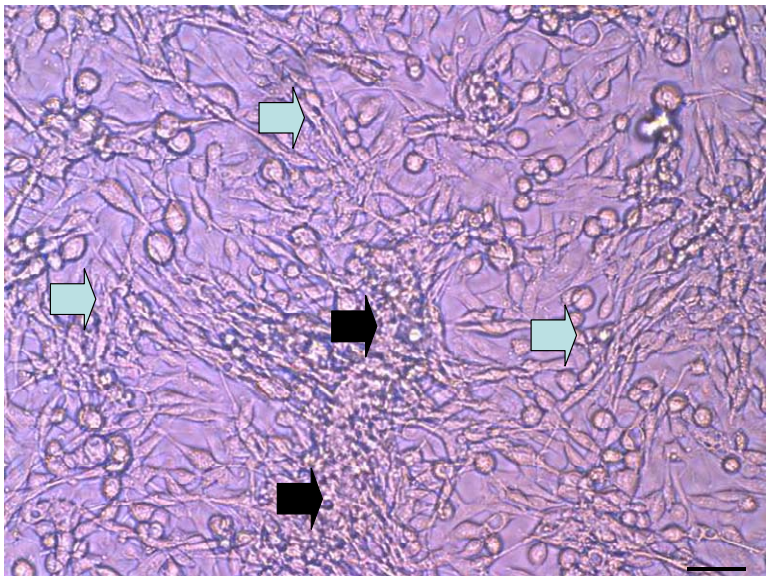


Plate34:Representative of B50 cells pre-treated at 0hrs with 10µM DAMGO against hypoxia for 96hrs of culture (5%O2 5% CO2), with few degenerated cells (Black arrow) and many normal cells (Blue arrow). The cells were observed in six different cultures with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

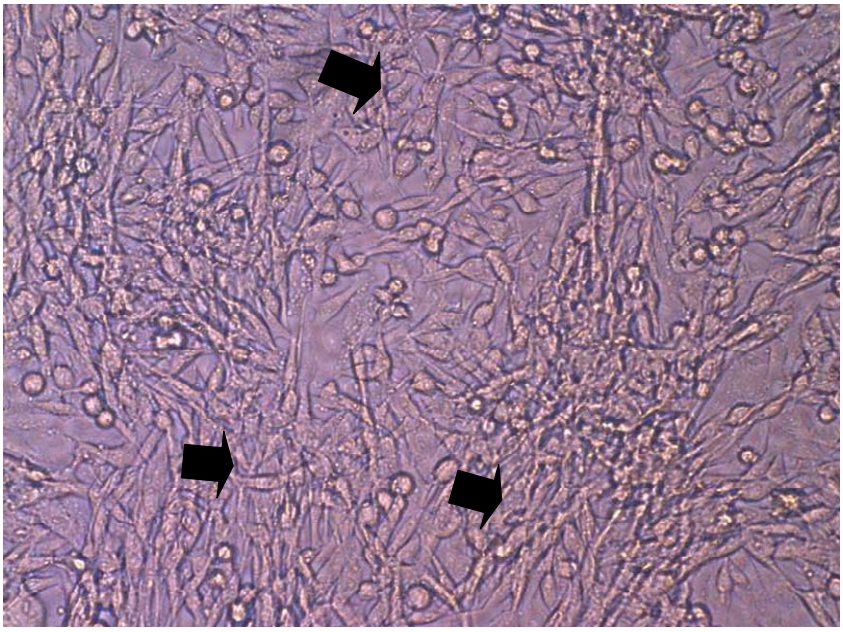
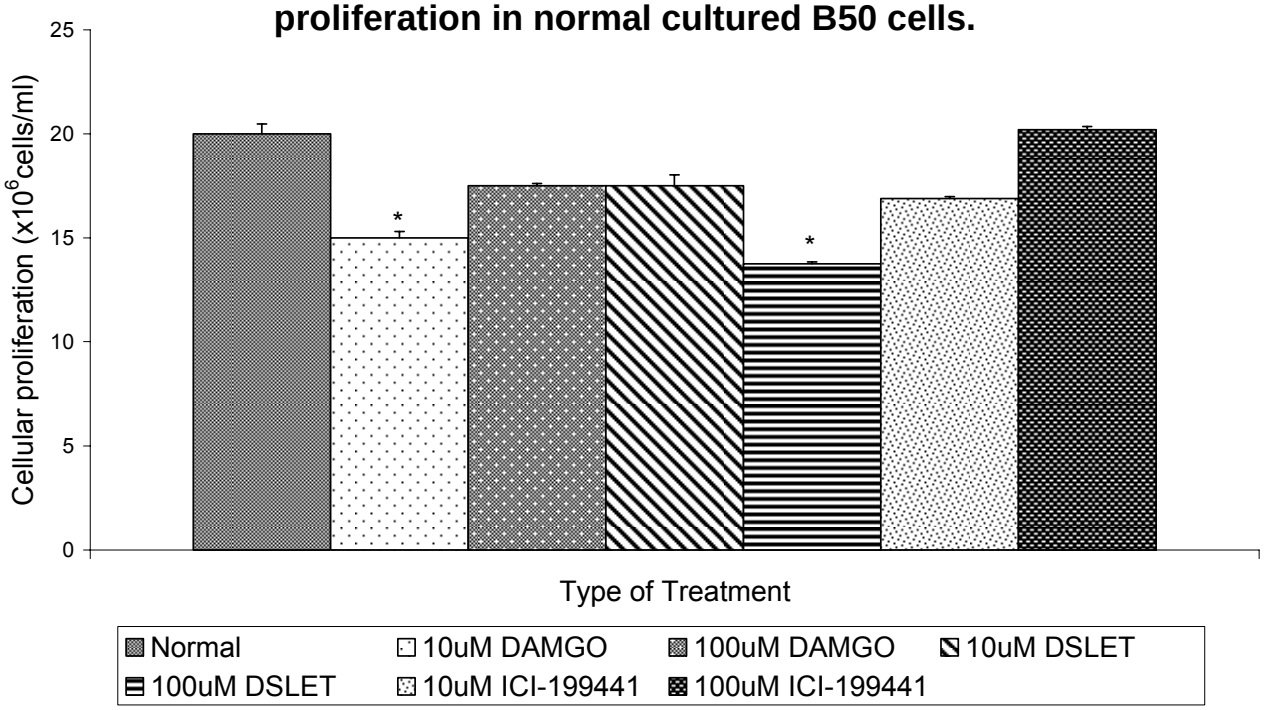


Plate35:Representative of B50 cells pre-treated at 0hrs with 10µM DSLET against hypoxia for 96hrs of culture (5%O2 5% CO2), with many normal cells (arrow). The cells were observed in six different cultures with same field method in a quadri-point analysis under the Nikon Eclipse TS100 microscope and microphotographs processed with an IBM Image Solutions®. Scale bar = 5mm x40 magnification.

3.7.2. The effect of opioid agonists on cellular proliferation in normal and hypoxic cultured B50 cells.

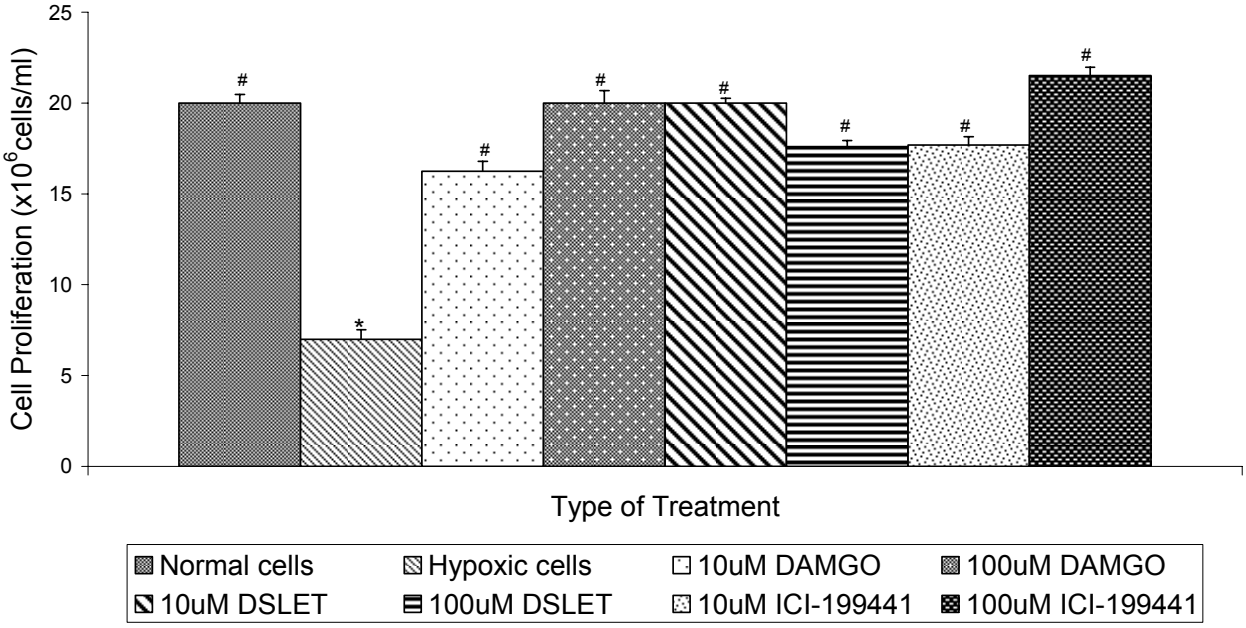
The effect of opioid agonist treatment on cellular proliferation in normal cultured B50 cells is shown in Figure 3.29. The result showed a significant decrease ($P<0.05$) in cell proliferation in cells treated with 10µM DAMGO ($15x10^6$cells/ml); 100µM DSLET ($13.75x10^6$cells/ml) and a non-significant decrease in proliferation in cells treated with 100µM DAMGO ($17.5x10^6$cells/ml) (P=0.73); 10µM DSLET ($17.5x10^6$ cells/ml)(P=0.62); and 10µM ICI-199441 ($16.88x10^6$cells/ ml)(P=0.56), when compared to untreated normal B50 cells ($20.20x10^6$ cells/ml). The results of the effects of opioid agonist treatment on cellular proliferation in cultured B50 cells in hypoxia, showed about the same pattern of proliferation between the cells treated with 100µM DAMGO ($20x10^6$cells/ml); 10µM DSLET ($20x10^6$cells/ml) and 100µM ICI-199441 ($21.52x10^6$cells/ml), when compared to untreated normal cells ($20x10^6$cells/ ml). There was a non-significant decrease in cells treated with 10µM DAMGO ($16.25x10^6$cells/ml) (P=0.66); 100µM DSLET ($17.6x10^6$cells/ml) (P=0.69) and 10µM ICI-199441 ($17.7x10^6$ cells/ml) (P=0.72), when compared to control cells ($20x10^6$cells /ml). However, the decrease in cellular proliferation was significant ($P<0.05$) when compared with the hypoxic untreated B50 cells ($7.0x10^6$cells/ml). When the effect of opioid agonist treatment on cellular proliferation was compared between the treated hypoxic B50 cells with different concentrations of opioid agonists and the untreated hypoxic cells ($7.0x10^6$cells/ml), the results showed a significant increase ($P<0.05$) in cellular proliferation in all treated B50 cell groups in hypoxia (Figure 3.30).

Fig.3.29.The effect of opioid agonist treatment on cellular proliferation in normal cultured B50 cells.



The effect of opioid agonist on cell proliferation of B50 cells cultured in normal ($21\%O_2;5\%CO_2$) conditions using proliferation assay method. The cells were cultured for 48hrs and then treated with the opioid agonists for 48hrs for a total of 96hrs of culture. The cells were quantified using standard curve prepared from trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing 42.5 x 10^6cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) x 10^6 cells/ml and the absorbance of the CellTiter assay (n=5) was measured at 490nm and plotted against the number of cells to give a standard curve. The number of cells from the tests was quantified using the CellTiter assay absorbance of the test groups against the known cell numbers from the standard.(Data presented as mean ±SD; n=6; *P<0.05 versus normal, untreated cells; Student's t-test).

Fig.3.30.The effect of opioid agonist treatment on cellular proliferation in cultured B50 cells in hypoxia

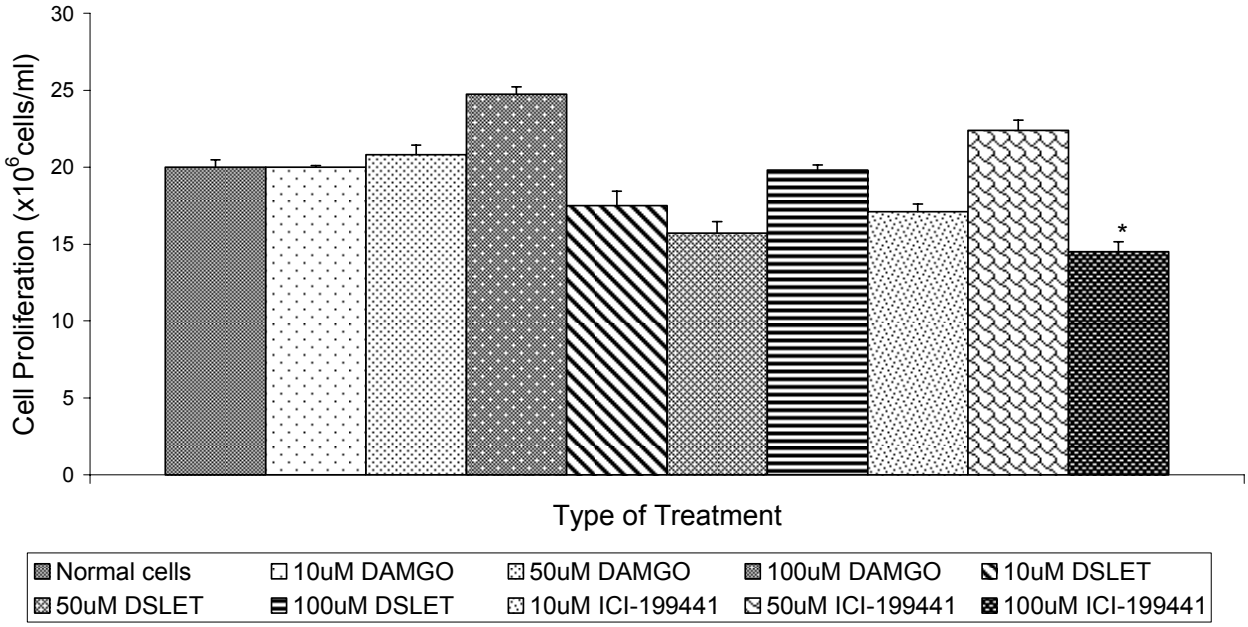


The effect of opioid agonist on cell proliferation of B50 cells cultured in hypoxic ($5\%O_2$; $5\%CO_2$) conditions using proliferation assay method. The cells were cultured for 48hrs and then treated with different opioid agonists at different concentrations for 48hrs for a total of 96hrs of culture. The number of proliferated cells was quantified using standard curve prepared from trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing 42.5 x 10^6cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) x10^6cells/ml and the absorbance of the CellTiter assay (n= 5) was measured at 490nm and plotted against the number of cells to give a standard curve. The number of cells from the tests was quantified using the absorbance of the test groups against the known cell numbers from the standard. The number of cells from untreated normal cultured cells ($21\%O_2$; $5\%CO_2$), was used as the control.(Data presented as mean ±SD; n=6; *$P<0.05$ versus normal, untreated cells; $^{\#}P<0.05$ versus hypoxia, untreated cells; Student's t-test).

The effect of concurrent culture and treatment of opioid agonist (pre-treatment) on cellular proliferation in normal cultured B50 cells showed that the B50 cells pre-treated with 10μM DAMGO ($20x10^6$cells/ml); 50μM DAMGO ($20.8x10^6$cells/ml) and 100μM DSLET ($19.8x10^6$cells/ml), had about the same cell proliferation pattern when compared with the normal untreated B50 cells ($20x10^6$cells/ml). There was a non-significant increase in cell proliferation in cells pre-treated with 100μM DAMGO ($24.75x10^6$cells/ml) (P=0.16) and 50μM ICI-199441 ($22.4x10^6$cells/ml) (P=0.09), a non-significant decrease in cell proliferation in cells pre-treated with 10μM DSLET ($17.5x10^6$cells/ml) (P=0.07), 50μM DSLET ($15.7x10^6$cells/ml) (P=0.18), 10μM ICI-199441 ($17x10^6$cells/ml) (P=0.082) and a significant decrease (P<0.05) in cellular proliferation in cells pre-treated with 100μM ICI-199441 ($14.5x10^6$cells/ml) when compared with the untreated normal cells ($20x10^6$cells/ml) in culture (Figure 3.31).

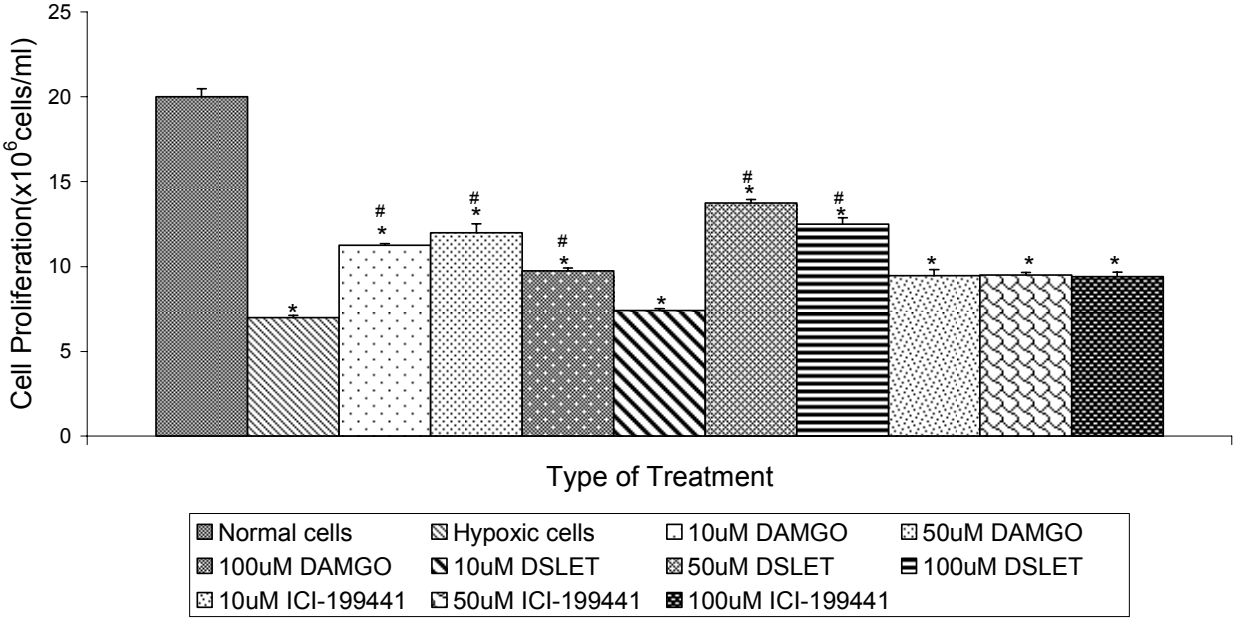
The effect of opioid agonist pre-treatment against hypoxia on cellular proliferation of B50 neuronal cells is shown in Figure 3.32. The result shows a significant decrease (P<0.05) in neuronal proliferation in untreated B50 cells cultured in hypoxia ($7.0x10^6$cells/ml) and other cells pre-treated with different concentrations of opioid agonist when compared to the untreated normal cells ($20x10^6$cells/ml). There was a significant increase (P<0.05) in cellular proliferation in B50 cells pre-treated with 10μM DAMGO ($11.25x10^6$cells/ml); 50μM DAMGO ($12.0x10^6$cells/ml); 100μM DAMGO ($9.75x10^6$cells/ml); 50μM DSLET ($13.75x10^6$cells/ml) and 100μM DSLET ($12.50x10^6$cells/ml) when compared with hypoxic untreated B50 cells in culture ($7x10^6$cells/ml) (Figure 3.32).

Fig.3.31.The effect of opioid agonist pre-treatment on cellular proliferation in cultured normal B50 cells



The effect of opioid agonist pre-treatment on cell proliferation of normal cultured B50 cells (21%O_2; 5%CO_2) using proliferation assay method. The cells were concurrently treated with different opioid agonists at 0hr and then cultured for a total of 96hrs. The number of proliferated cells was quantified using standard curve prepared from trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing 42.5 x 10^6cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) x 10^6cells/ml and the absorbance of the CellTiter assay (n= 5) was measured at 490nm and plotted against the number of cells to give a standard curve. The number of cells from the tests was quantified using the absorbance of the test groups against the known cell numbers from the standard.(Data presented as mean ±SD; n=6; *P<0.05 versus normal, untreated cells; Student's t-test).

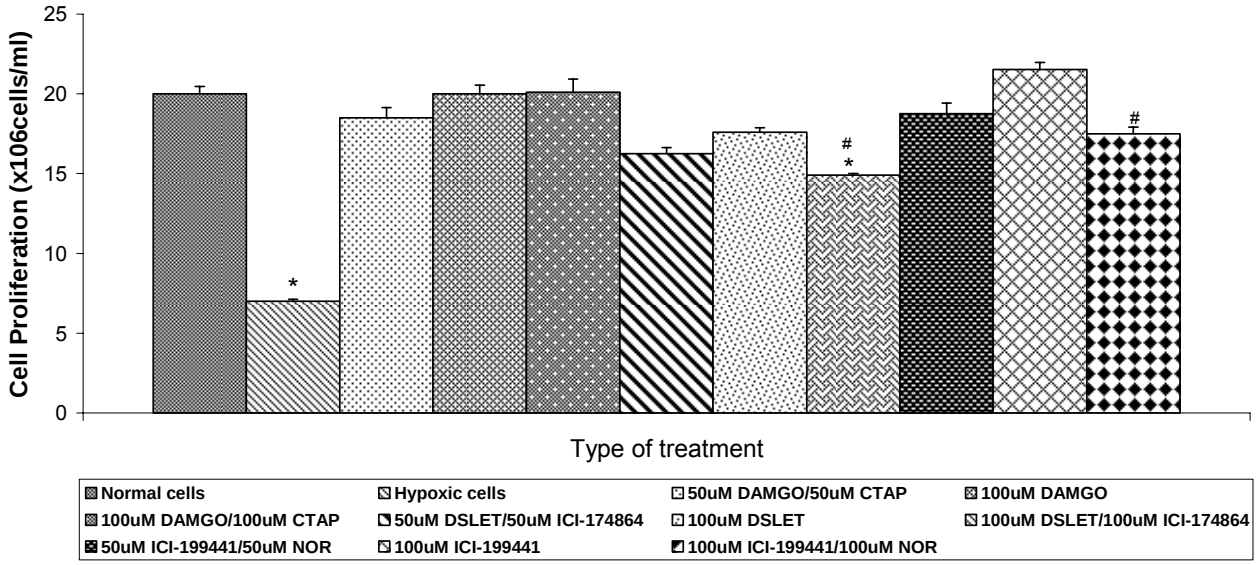
Fig.3.32.The effect of opioid agonist pre-treatment on cellular proliferation in cultured B50 cells in hypoxia



The effect of opioid agonist pre-treatment against hypoxia on cell proliferation of B50 cells cultured in hypoxic ($5\%O_2$; $5\%CO_2$) conditions using proliferation assay method. The cells were concurrently treated with different opioid agonists and exposed to hypoxia at 0hr and then cultured for a total of 96hrs. The number of proliferated cells was quantified using standard curve prepared from trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing 42.5 x 10^6cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) x 10^6 cells/ml and the absorbance of the CellTiter assay (n= 5) was measured at 490nm and plotted against the number of cells to give a standard curve. The number of cells from the tests was quantified using the absorbance of the test groups against the known cell numbers from the standard. The untreated normal cells ($21\%O_2$; $5\%CO_2$), was used as the control. (Data presented as mean ±SD; n=6; *$P<0.05$ versus normal, untreated cells; $^{\#}P<0.05$ versus hypoxia, untreated cells; Student's t-test).

The result of opioid agonist treatment in the presence of antagonist on cellular proliferation in cultured B50 cells in hypoxia, shows similar trends in untreated normal B50 cells ($20x10^6$cells/ml) and cells treated with 100µM CTAP/100µM DAMGO, while the cells treated with different concentrations of opioid agonists and antagonists showed different levels of non-significant decreases in cell proliferation from the untreated normal cells. The results are 50µM CTAP/50µM DAMGO $18.5x10^6$cells/ml (P=0.19); 50µM ICI-174864/50µM DSLET $16x10^6$cells/ml (P=0.09); 50µM NOR/ 50µM ICI-199441 $18.7x10^6$cells/ml (P=0.21); 100µM NOR/100µM ICI-199441 $17.5x10^6$cells/ml (P=0.11) and a significant decrease ($P<0.05$) in cellular proliferation from 100µM ICI-174864/100µM DSLET ($14.9x10^6$ cells/ml) when compared to the proliferation of the untreated normal cells ($20x10^6$cells/ml). The results show a significant decrease ($P<0.05$) in cellular proliferation in B50 cells treated with agonist/antagonist in 100µM DSLET/100µM ICI-174864 ($14.9x10^6$cells/ml) and 100µM ICI-1999441/100µM NOR ($17.5x10^6$cells /ml) when compared to that of hypoxic agonist treated B50 cells (Figure 3.33).

Fig.3.33.The effect of opioid agonist and antagonist treatment on cellular proliferation in cultured B50 cells in hypoxia.



The effect of opioid agonist treatment in the presence of antagonist on cell proliferation of B50 cells cultured in hypoxic ($5\%O_2$;$5\%CO_2$) conditions using proliferation assay method. The cells were cultured for 48hrs and then treated with the opioid agonists and antagonist for another 48hrs for a total of 96hrs of culture. The number of proliferated cells was quantified using standard curve prepared from trypan blue counting method and CellTiter Aqueous One Solution assay method. Culture plate containing 42.5 x 10^6 cells/ml was used to prepare five dilutions (0, 2, 4, 5 and 10) x 10^6 cells/ml and the absorbance of the CellTiter assay (n=5) was measured at 490nm and plotted against the number of cells to give a standard curve. The number of cells from the tests was quantified using the absorbance of the test groups against the known cell numbers from the standard. The untreated normal cultured cells ($21\%O_2$; $5\%CO_2$) was used as the control.(Data as mean ±SD; n=6; *$P<0.05$ versus normal, untreated cells; $^{\#}P<0.05$ versus agonist/antagonist treated cells; Student's t-test).

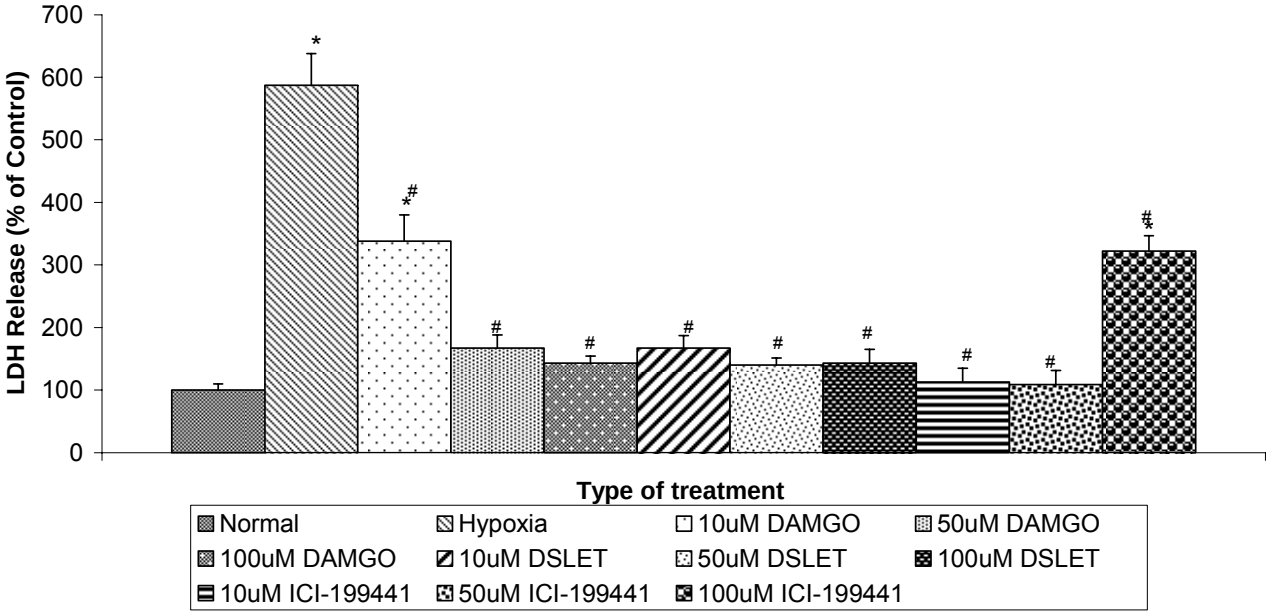
3.8 Opioid receptor agonist effect on LDH

3.8.1 LDH release from treatment with opioid agonists ± antagonists in B50 neuronal cells cultured in hypoxia

The LDH leakage from opioid agonists treated B50 neuronal cells in hypoxia is shown in Figure 3.34. The results show a significant 5-fold increase in LDH release ($P<0.05$) from untreated hypoxic B50 cells (587%) when compared with normal cells (100%), and a non-significant increase in LDH release from cells treated with 100µM DAMGO (143%) ($P=0.51$); 50µM DSLET (140%) ($P=0.58$); 100µM DSLET (143%) ($P=0.55$); 10µM ICI-199441 (113%) ($P=0.64$) and 50µM ICI-199441 (109%) ($P=0.81$). The results of the treatment with opioid agonists showed a significant 3-to 5-fold decrease ($P<0.05$) in LDH release in cells treated with 10µM DAMGO (338%), 100µM ICI-199441 (322%), 100µM DAMGO (143%); 50µM DSLET (140%); 100µM DSLET (143%);10µM ICI-199441 (113%) and 50µM ICI-199441 (109%), when compared with the untreated hypoxic cells (Figure 3.34).

The results showed approximately, between a 2- and 8-fold increase in LDH leakage from hypoxia untreated cells and cells treated with opioid agonists/antagonists when compared to the LDH release from the normal B50 cells. When the LDH release from opioid agonist treated hypoxic cells was compared with cells treated with opioid agonist/antagonist, the results showed a significant increase ($P<0.05$) in LDH release from 100µM DAMGO (143%) and 100µM DAMGO/100µM CTAP (364%); 50µM DSLET (140%) and 50µM DSLET/50µM ICI-174864 (416%); 10µM ICI-1999441 (113%) and 10µM ICI-199441/10µM NOR (821%); 100µM ICI-1999441(322%) and 100µM ICI-199441/100µM NOR (665%) (Figures 3.35a, b and c).

Fig.3.34.The effect of opioid treatment on LDH release in B50 cells culture in hypoxia



The effect of opioid agonist treatment on LDH release from B50 cells cultured in hypoxia (5%O_2; 5%CO_2), using LDH assay. Cells were cultured for 48hrs and then treated with different concentrations of opioid agonists for 48hrs for a total of 96hrs of culture. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells (21%O_2;5%CO_2) was normalized and used as the control (100%) and the LDH release was expressed relative to the control. (Data as mean ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus hypoxic untreated cells; Student's t-test).

Fig.3.35a.The effect of opioid treatment on LDH release in B50 cells cultured in hypoxia

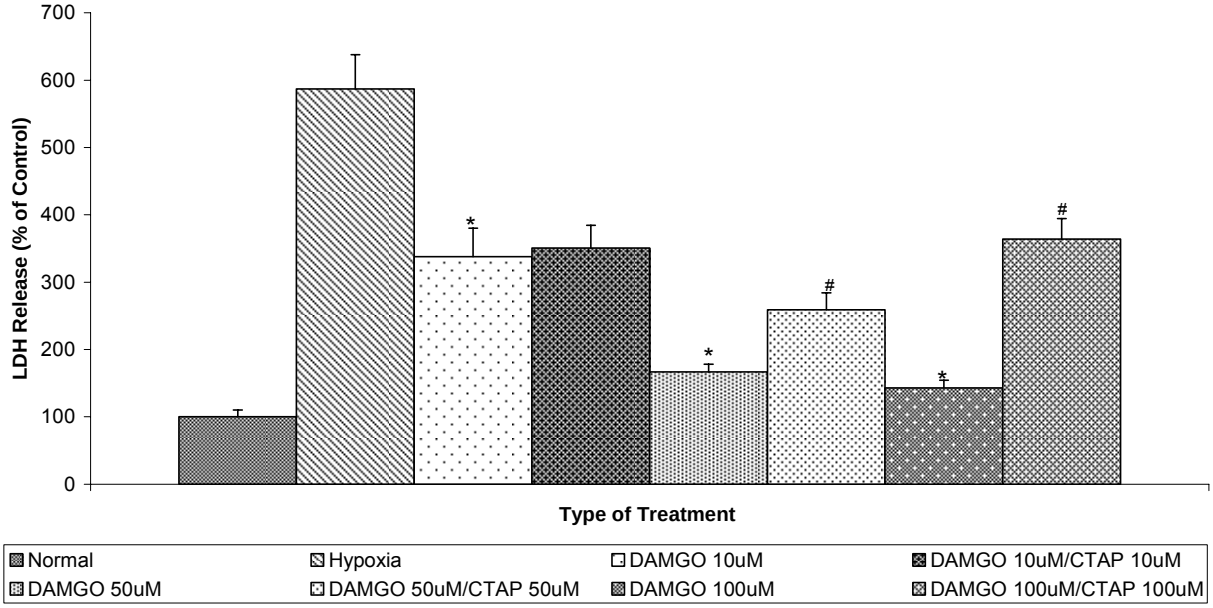
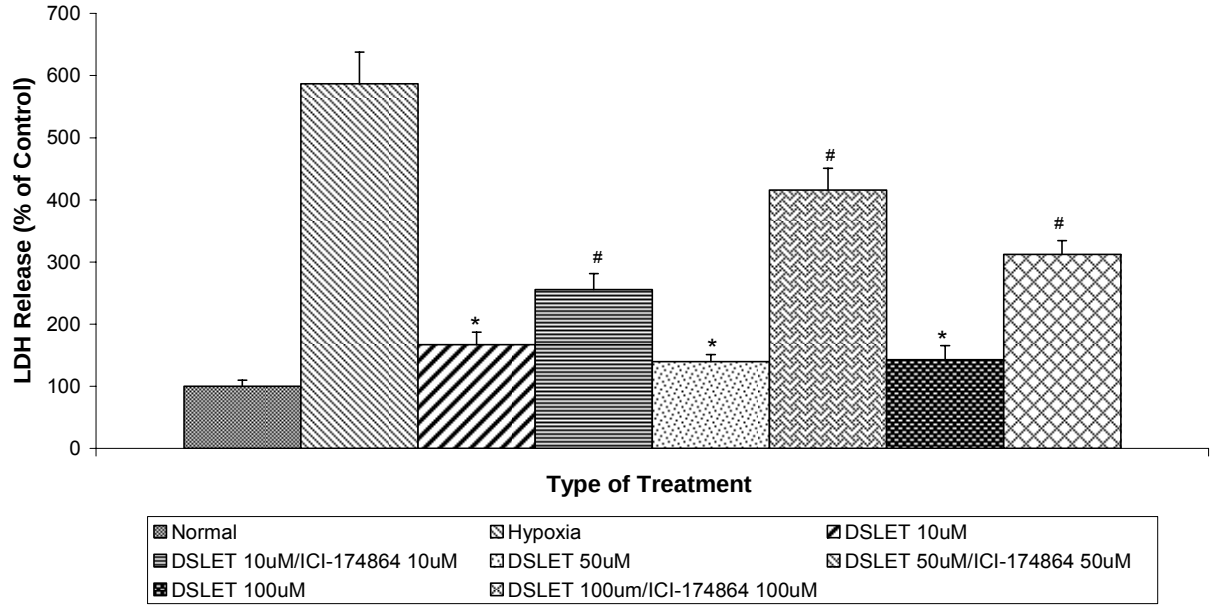
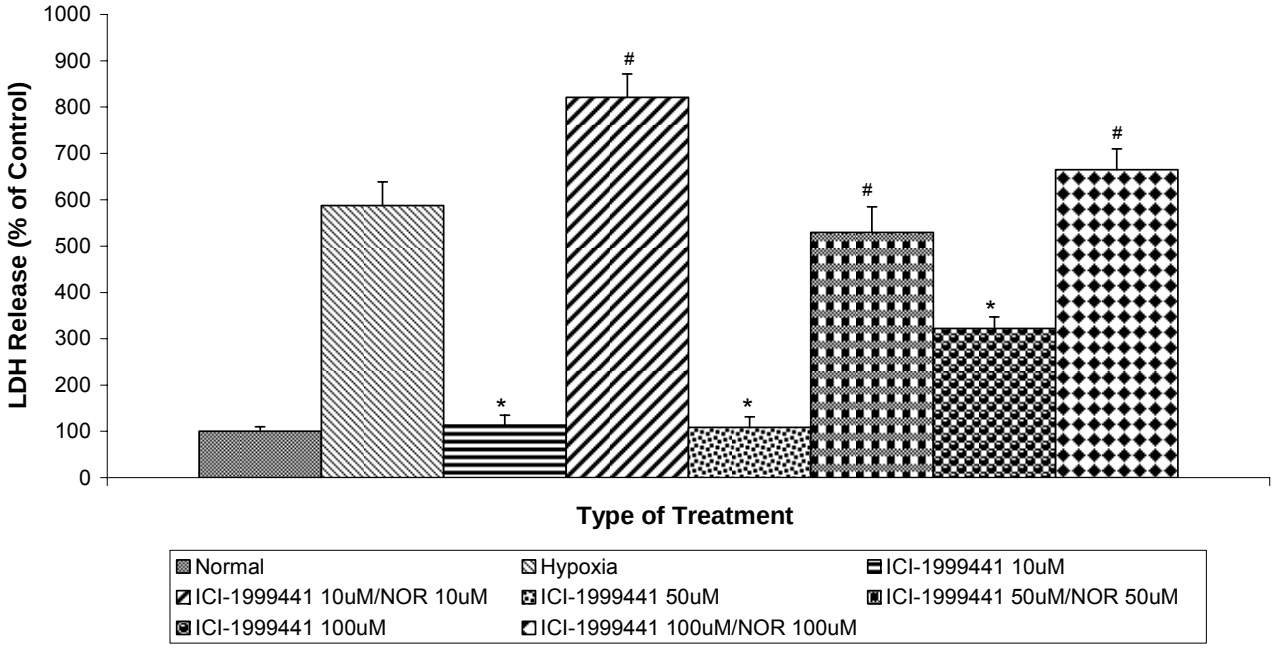


Fig.3.35b.The effect of opioid treatment on LDH release in B50 cells cultured in hypoxia.



Figs.3.35a & b.The effect of opioid agonist/antagonist treatment on LDH release from B50 cells cultured in hypoxia ($5\%O_2$; $5\%CO_2$), using LDH assay. Cells were cultured for 48hrs and then treated with different concentrations of opioid agonists for 48hrs for a total of 96hrs of culture. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells ($21\%O_2$;$5\%CO_2$) was normalized and used as the control (100%) and the LDH release was expressed relative to the control.(Data as mean ±SD; *$P<0.05$ versus untreated hypoxic cells; $^{\#}P<0.05$ versus agonist/antagonist treated hypoxic cells; Student's t-test).

Fig.3.35c.The effect of opioid treatment on LDH release in B50 cells cultured in hypoxia.



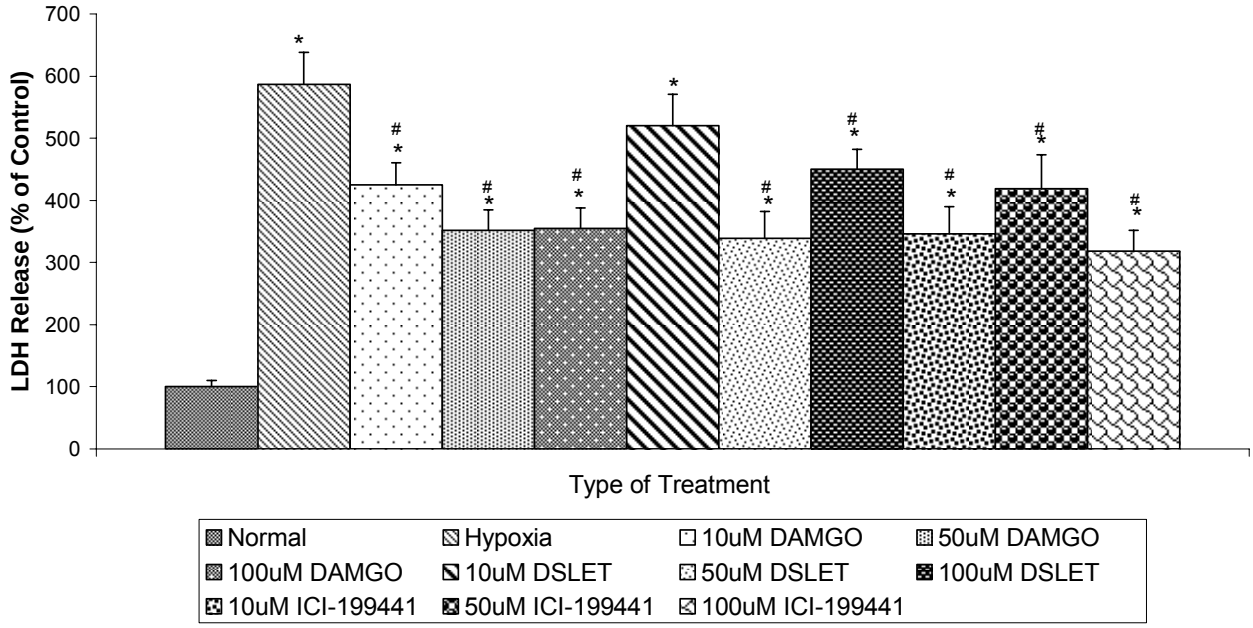
The effect of opioid agonist/antagonist treatment on LDH release from B50 cells cultured in hypoxia (5%O_2; 5%CO_2), using LDH assay. Cells were cultured for 48hrs and then treated with different concentrations of opioid agonists for 48hrs for a total of 96hrs of culture. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells (21%O_2;5%CO_2) was normalized and used as the control (100%) and the LDH release was expressed relative to the control.(Data as mean ±SD; *P<0.05 versus untreated hypoxic cells; $^{\#}$P<0.05 versus agonist/antagonist treated hypoxic cells; Student's t-test).

3.8.2 LDH Release from opioid agonists and antagonist pre-treated B50 cells cultured in hypoxia.

The effect of opioid agonist pre-treatment against hypoxia showed a significant 3- to 5-fold increase in LDH release ($P<0.05$) from untreated hypoxic B50 cells (587%), and the hypoxic cells pre-treated with different concentrations of opioid agonists are; 10μM DAMGO (425%), 50μM DAMGO (352%), 100μM DAMGO (355%), 10μM DSLET (520%), 50μM DSLET (339%), 100μM DSLET (450%), 10μM ICI-199441 (346%), 50μM ICI-199441 (419%) and 100μM ICI-199441 (318%) when compared to normal B50 cells (100%). The results showed a significant 2- to 3-fold decreases in LDH release ($p<0.05$) in cells pre-treated with different concentrations of opioid agonist when compared with the untreated hypoxic cells in culture (Figure 3.36)

The LDH release from hypoxic B50 cells pre-treated with opioid agonists in the presence of antagonists, showed a significant 2-to 6-fold increase in LDH release ($p<0.05$) from untreated hypoxic B50 cells (587%) and cells pre-treated with different concentrations of opioid agonists/antagonists when compared to the normal B50 cells (100%). When the result of the LDH release from hypoxic pre-treated cells was compared with cells pre-treated with the different concentrations of the opioid agonist/antagonist, the result showed a significant increase ($p<0.05$) in LDH release between 50μM DAMGO (352%) and 50μM DAMGO/50μM CTAP (690%); 50μM DSLET (339%) and 50μM DSLET/50μM ICI-174864 (611%), 50μM ICI-1999441 (419%) and 50μM ICI-199441/50μM NOR (619%).(Figures 3.37a, b and c).

Fig.3.36.The effect of opioid agonist pre-treatment on LDH release in B50 cells cultured in hypoxia.



The effect of opioid agonist pre-treatment against hypoxia on LDH release from B50 cells in hypoxic culture (5%O_2; 5%CO_2), using LDH assay. Cells were concurrently treated with different concentrations of opioid agonists and exposed to hypoxia at 0hr and cultured for 96hrs. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells (21%O_2;5%CO_2) was normalized and used as the control (100%) and LDH release was expressed relative to the control.(Data as means ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus hypoxic untreated cells; Student's t-test).

Fig.3.37a.The effect of opioid agonist/antagonist pre-treatment on LDH release in cultured B50 cells in hypoxia.

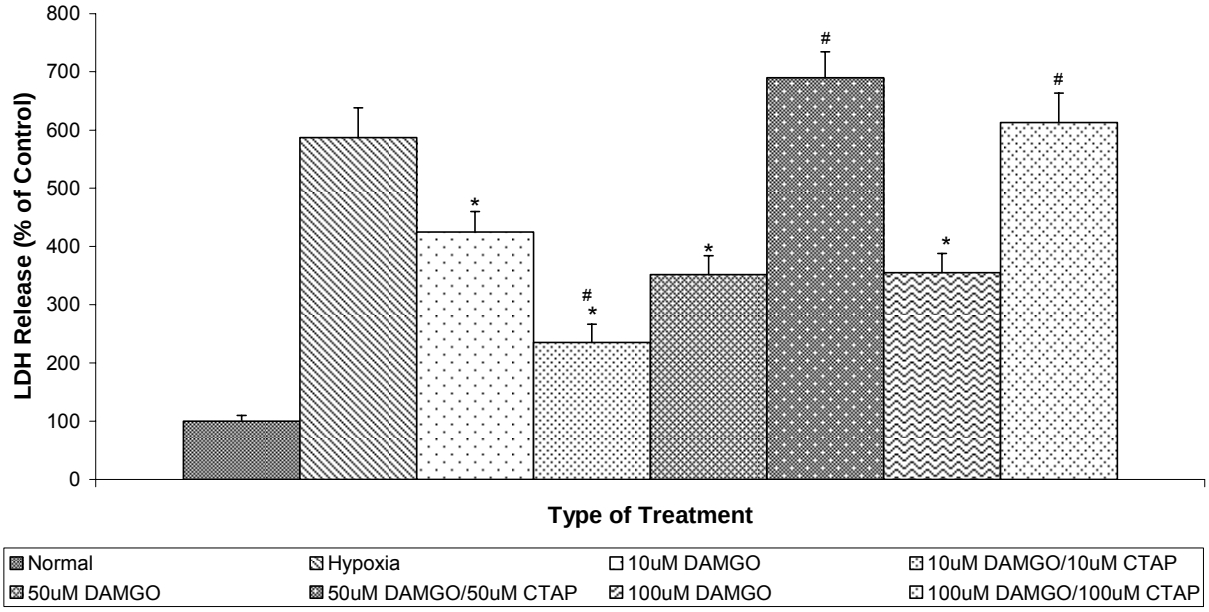
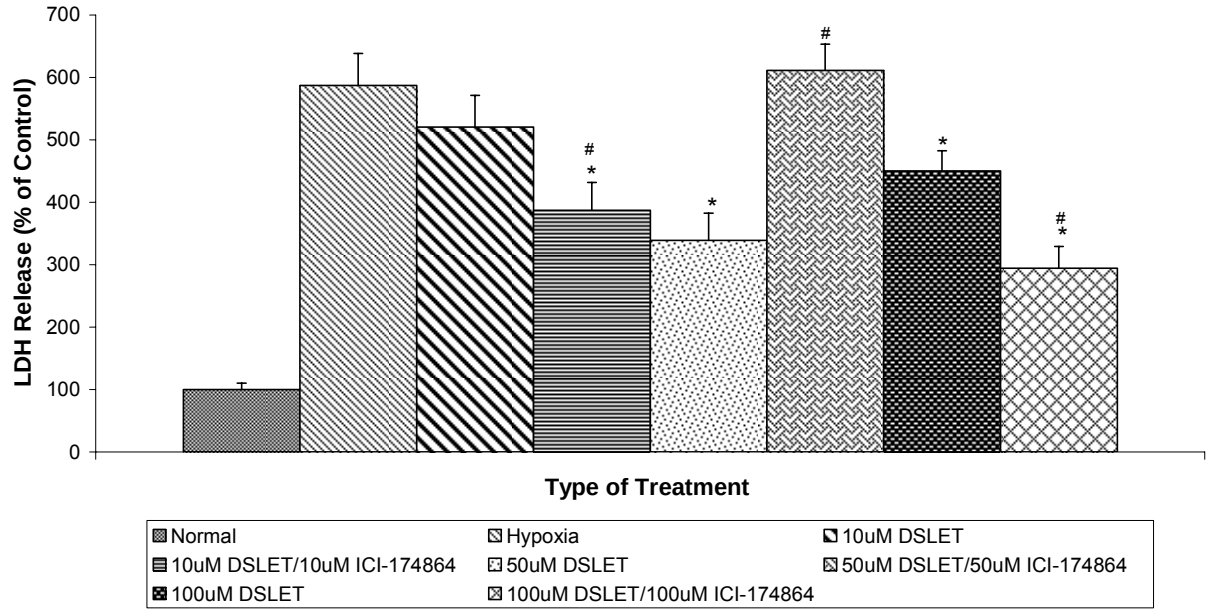
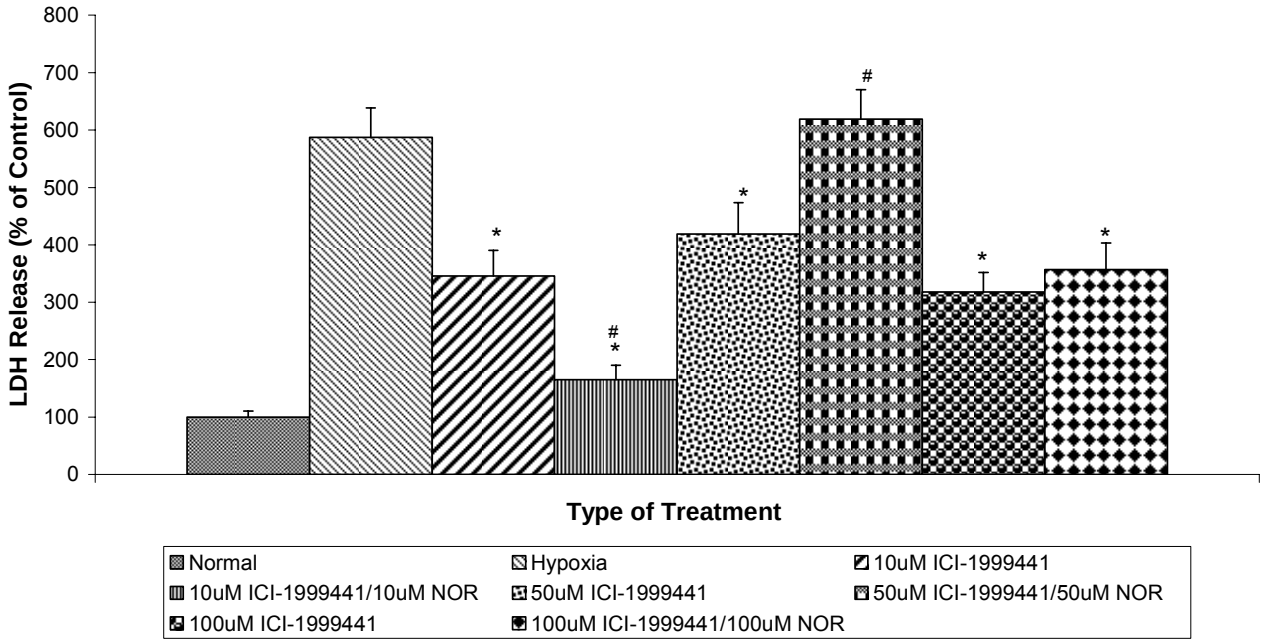


FIG.3.37b.The effect of opioid agonist/antagonist pre-treatment on LDH release in cultured B50 cells in hypoxia



Figs.3.37a & b:The effect of opioid agonist and antagonist pre-treatment against hypoxia on LDH release from B50 cells cultured in hypoxia ($5\%O_2$; $5\%CO_2$), using LDH assay. Cells were treated concurrently with different concentrations of opioid agonists/antagonists and exposed to hypoxia at 0hr and then cultured for 96hrs. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells ($21\%O_2$;$5\%CO_2$) was normalized and used as the control (100%) and the LDH release was expressed relative to the control.(Data as mean ±SD; *$P<0.05$ versus hypoxic untreated cells; $^{\#}P<0.05$ versus agonist/antagonist treated cells; Student's t-test).

Fig.3.37c.The effect of opioid agonist/antagonist pre-treatment on LDH release in cultured B50 cells in hypoxia.



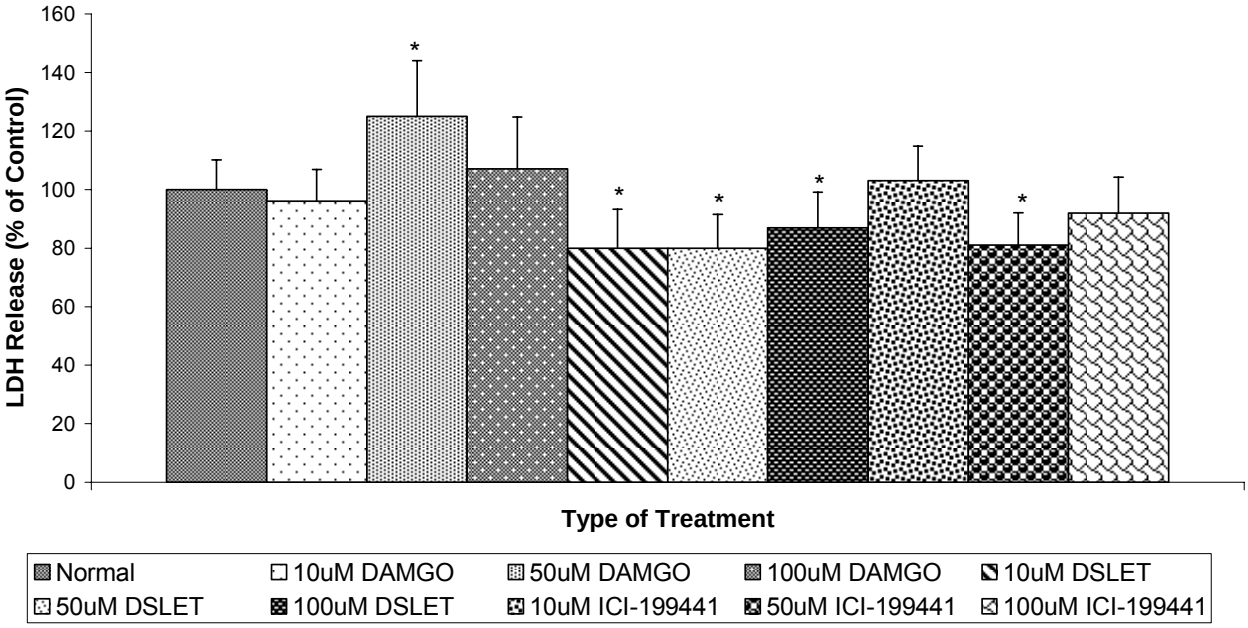
The effect of opioid agonist and antagonist pre-treatment against hypoxia on LDH release from B50 cells cultured in hypoxia (5%O_2; 5%CO_2), using LDH assay. Cells were treated concurrently with different concentrations of opioid agonists/antagonists and exposed to hypoxia at 0hr and then cultured for 96hrs. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells (21%O_2;5%CO_2) was normalized and used as the control (100%) and the LDH release was expressed relative to the control.(Data as mean ±SD; *P<0.05 versus hypoxic untreated cells; $^{\#}$P<0.05 versus agonist/antagonist treated cells; Student's t-test).

3.8.3. LDH Release from normal cultured B50 cells treated and pre-treated with opioid agonists.

The effect of opioid agonist treatment on LDH leakage from normal B50 cells in culture, showed a significant reduction ($P<0.05$) in LDH release in B50 cells treated with 10µM DSLET (80%), 50µM DSLET (80%), 50µM ICI-199441 (81%) and 100µM DSLET (87%); a significant increase ($P<0.05$) in cells treated with 50µM DAMGO (125%), and an insignificant decrease in LDH release in cells treated with 100µM ICI-199441 (92%) ($P=0.18$) and 10µM DAMGO (96%) ($P=0.09$). The cells treated with 100µM DAMGO (107%) ($P=0.08$) and 10µM ICI-199441 (103%) ($P=0.07$), showed a non-significant increase in LDH release when compared with the untreated normal B50 cells in culture (100%). (Figure 3.38)

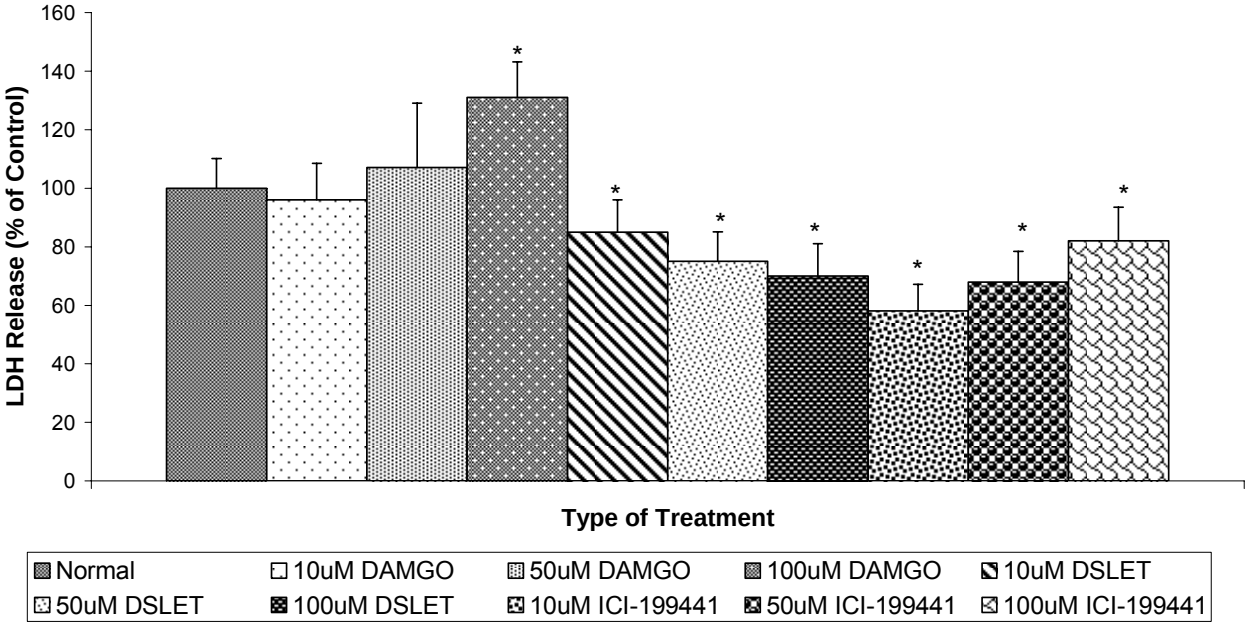
The effect of opioid agonist pre-treatment on LDH release from normal B50 cells in culture, showed a significant increase ($P<0.05$) in LDH release from B50 cells pre-treated with 100µM DAMGO (131%) and a non-significant increase in LDH release in cells pre-treated with 50µM DAMGO (107%) ($P=0.11$), when compared with the untreated normal cells (100%). There was a significant decrease ($P<0.05$) in LDH release from B50 cells pre-treated with 50µM DSLET (75%), 100µM DSLET (70%), 10µM ICI-199441 (59%), 50µM ICI-199441 (68%), 100µM ICI-199441 (82%), 10µM DSLET (85%), and a non-significant decrease in LDH release in cells with 10µM DAMGO (96%) ($P=0.07$) when compared with the untreated normal B50 cells (100%) in culture (Figure 3.39).

Fig.3.38.The effect of opioid treatment on LDH release in normal B50 cells



The effect of opioid agonist treatment on LDH release from normal B50 cells in culture using LDH assay. Cells were cultured in normal ($21\%O_2$; $5\%CO_2$) conditions for 48hrs and then treated with different concentrations of opioid agonists for 48hrs for a total of 96hrs of culture time. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells was normalized and used as the control (100%) and the LDH release was expressed relative to the control.(Data as mean ±SD; *$P<0.05$ versus untreated normal cells; Student's t-test).

Fig.3.39.The effect of opioid pre-treatment on LDH release in normal B50 cells



The effect of opioid agonist pre-treatment on LDH release from normal B50 cells in culture using LDH assay. Cells were cultured in normal ($21\%O_2;5\%CO_2$) conditions and treated at 0hr with different concentrations of opioid agonists and cultured for 96hrs in culture. The absorbance of the assay (n=6) was measured at 490nm. The LDH released from the untreated normal cultured cells was normalized and used as the control (100%) for the experiment and LDH release was expressed relative to the control.(Data as mean ±SD; *P<0.05 versus untreated normal cells; Student's t-test).

3.9 Effect of hypoxia on signalling molecules

3.9.1 The effect of hypoxia on cAMP levels in B50 cells treated with cannabinoid agonists.

The effect of hypoxia on cAMP levels in B50 cells treated with cannabinoid agonists (Win, AEA and 2-AG) is shown in Figure 3.40. The result showed an inverse relationship between the percentage bound cAMP and the amount of free cAMP released into the B50 cells in picomoles per ml as in Figure 3.41. The result showed that the untreated normal cells had 3.0pmol/ml of free cAMP while the untreated hypoxic cells had 0.70pmol/ml of free cAMP. The hypoxic treated cells with different concentrations of cannabinoid agonists had cAMP release as shown in Table 3.18. When intracellular cAMP release in untreated hypoxic B50 cells was compared to the hypoxic treated B50 cells with different concentrations of cannabinoid agonists, the results showed a significant increase ($P<0.05$) in cAMP levels in the treated cells except for cells treated with 10nM AEA (0.75pmol/ml) and 100nM 2-AG (0.9pmol/ml) where the increase was not significant. There was a non-significant decrease in cAMP from cells treated with 50nM AEA (0.50pmol/ml) (Table 3.18).

Table 3.18: The effect of hypoxia on cAMP levels in B50 cells treated with cannabinoid agonists.

Type of Treatment	Net Optical Density (NoD)	Percent Bound (% Bound)	cAMP (pmol/ml)
Normal cells	0.292±0.163	57.78	3.0
Hypoxia no Drug	0.391±0.133	77.50	0.70*
Hypoxia/10nM Win	0.305±0.141	60.37	1.10*#
Hypoxia/50nM Win	0.346±0.090	68.58	1.0*#
Hypoxia/100nM Win	0.261±0.119	51.64	3.3#
Hypoxia/10nM AEA	0.390±0.047	77.30	0.75*
Hypoxia/50nM AEA	0.416±0.225	82.36	0.50*
Hypoxia/100nM AEA	0.348±0.156	68.89	1.00*#
Hypoxia/10nM 2-AG	0.162±0.121	51.83	3.50#
Hypoxia/50nM 2-AG	0.285±0.195	56.39	3.10#
Hypoxia/100nM 2-AG	0.350±0.059	69.28	0.90*

The effect of hypoxia on cAMP levels in B50 cells treated with cannabinoid agonist in hypoxia. Cells were cultured for 48hrs in hypoxia ($5\%O_2;5\%CO_2$) and then treated for 48hrs with different concentrations of different cannabinoid agonists for a total of 96hrs of culture. The absorbance of the cAMP assay (n=6) was measured at a wavelength of 410nm. cAMP from the untreated normal ($21\%O_2;5\%CO_2$) B50 cells was used as the control (Data presented as mean ±SD; *$P<0.05$ versus untreated normal cells; #$P<0.05$ versus hypoxia untreated cells; Student's t-test).

Fig.3.40. The effect of cannabinoid agonist treatment on intracellular cAMP levels in B50 cells in hypoxia.

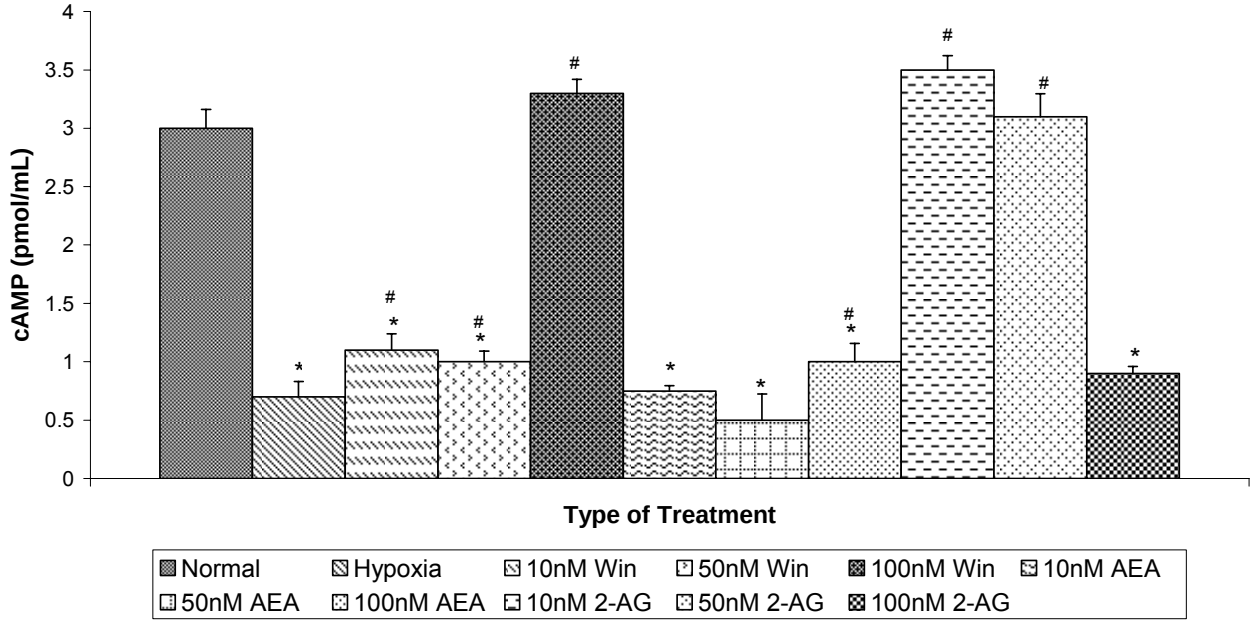
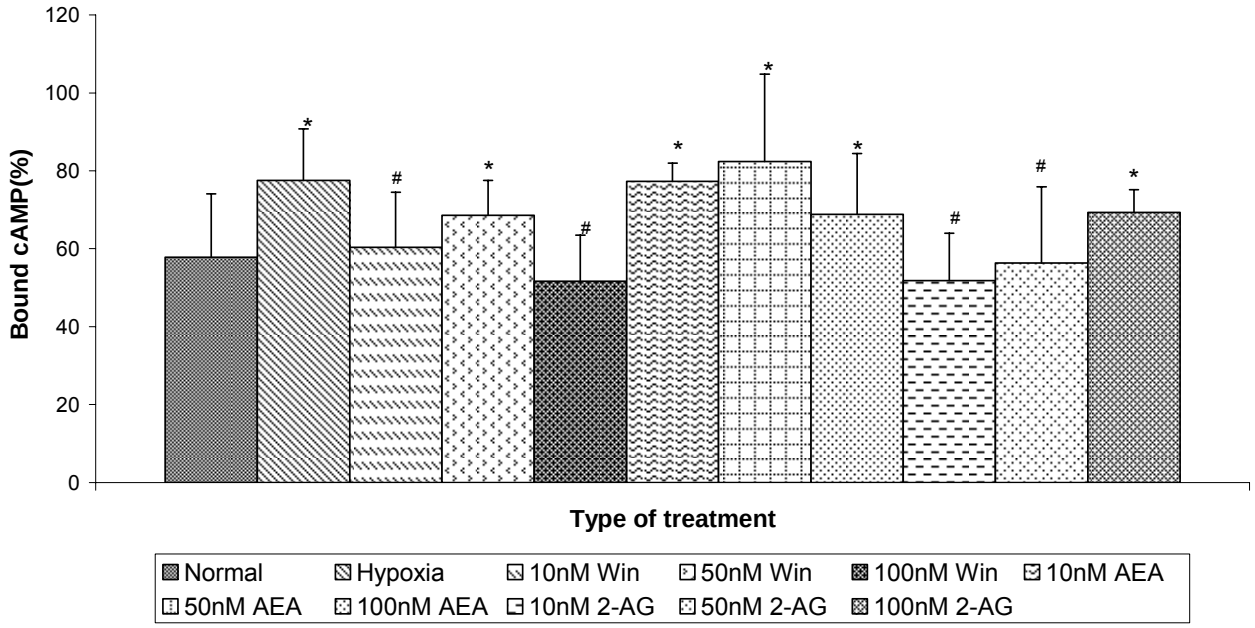


Fig.3.41. The effect of cannabinoid agonist treatment on bound cAMP levels in B50 cells in hypoxia



The effect of cannabinoid agonist treatment on intracellular and bound cAMP in B50 cells in hypoxia, as estimated by cAMP immunoassay. Cells were cultured for 48hrs in hypoxia ($5\%O_2$; $5\%CO_2$) and then treated for 48hrs with different concentrations of cannabinoid agonists for a total of 96hrs of culture. The absorbance of the cAMP assay (n=6) was measured at a wavelength of 410nm. The intracellular cAMP from the untreated normal ($21\%O_2$;$5\%CO_2$) cultured B50 cells was used as the control (Data presented as mean ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus hypoxia untreated; Student's t-test).

3.9.2. The effect of hypoxia on cAMP levels in B50 cells treated with opioid agonists.

The effect of hypoxia on cAMP levels in B50 cells treated with opioid agonists is shown in Figure 3.42. The results showed that the normal cultured B50 cells had 3.0 pmol/ml of cAMP released into the cytoplasm while the untreated hypoxic cells had 0.70 pmol/ml of cAMP released. The cAMP releases following treatment with the different concentrations of opioid agonists are shown in Table 3.19. The result showed a significant increase ($P<0.05$) in intracellular cAMP release in hypoxic B50 cells treated with 10μM ICI-199441 (10.0pmol/ml), and a non-significant increase in cAMP release in cells treated with 10μM DSLET (3.25pmol/ml); 50μM DSLET (3.25pmol/ml); 100μM DSLET (3.20pmol/ml) and 50μM ICI-199441 (3.15pmol/ml) when compared with the control (3.0pmol/ml). There was a significant decrease ($P<0.05$) in cAMP release from 10μM DAMGO (2.5pmol/ml); 100μM DAMGO (1.5pmol/ml); 100μM ICI-199441 (1.15pmol/ml) and hypoxia untreated cells (0.70pmol/ml) and a significant increase ($P<0.05$) in cAMP release in all the opioid treated cells in hypoxia when compared with the untreated hypoxic cells in culture. The result showed an inverse relationship between the bound intracellular cAMP and the released intracellular cAMP in the neuronal B50 cells (Figure 3.43). The result showed that the more the intracellular cAMP is bound, the less the intracellular cAMP released into the cytoplasm for cellular signalling activities while the less the cAMP that is bound, the more the cAMP that is released into the cytoplasm of the B50 cells.

Table 3.19: The effect of hypoxia on cAMP levels in B50 cells treated with opioid Agonists

Type of Treatment	Net Optical Density (NoD)	Percent Bound (% Bound)	cAMP (Pmol/ml
Normal Cells	0.292±0.163	57.78	3.00
Hypoxia no Drug	0.391±.0.133	77.50	0.70*
Hypoxia/10µM DAMGO	0.296±0.263	58.57	2.50*#
Hypoxia/50µM DAMGO	0.279±0.119	55.20	3.00#
Hypoxia/100µM DAMGO	0.316±0.026	62.54	1.50*#
Hypoxia/10µM DSLET	0.265±0.155	52.43	3.25#
Hypoxia/50µM DSLET	0.267±0.149	52.83	3.25#
Hypoxia/100µM DSLET	0.268±0.054	53.02	3.20#
Hypoxia/10µM ICI-199441	0.166±0.169	32.81	10.00*#
Hypoxia/50µM ICI-199441	0.270±0.157	53.42	3.15#
Hypoxia/100µM ICI-199441	0.319±0.062	63.13	1.15*#

Direct cAMP immunoassay in B50 cells treated with opioid agonists in hypoxia. Cells were cultured for 48hrs in hypoxia ($5\%O_2;5\%CO_2$) and then treated for 48hrs with different concentrations of different opioid agonists for a total of 96hrs of culture. The absorbance of the cAMP assay (n=6) was measured at a wavelength of 410nm. cAMP from the untreated normal ($21\%O_2;5\%CO_2$) B50 cells was used as the control (Data presented as mean ±SD; *$P<0.05$ versus untreated normal cells; #$P<0.05$ versus hypoxia untreated; Student's t-test).

Fig.3.42.The effect of opioid agonist on intracellular cAMP levels in cultured B50 cells in hypoxia.

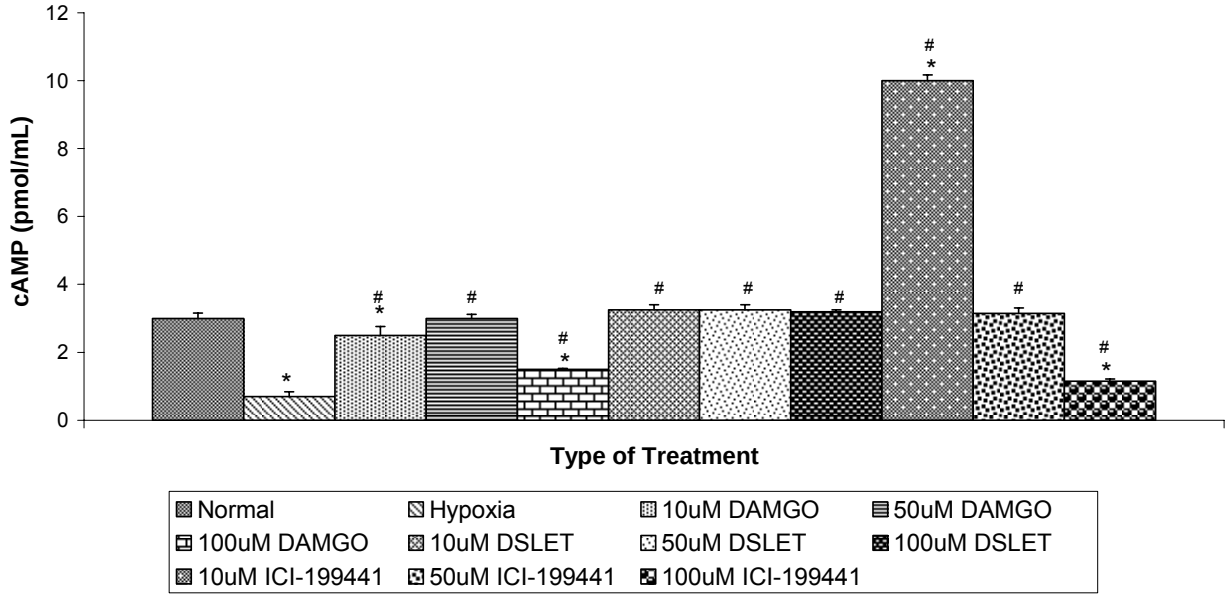
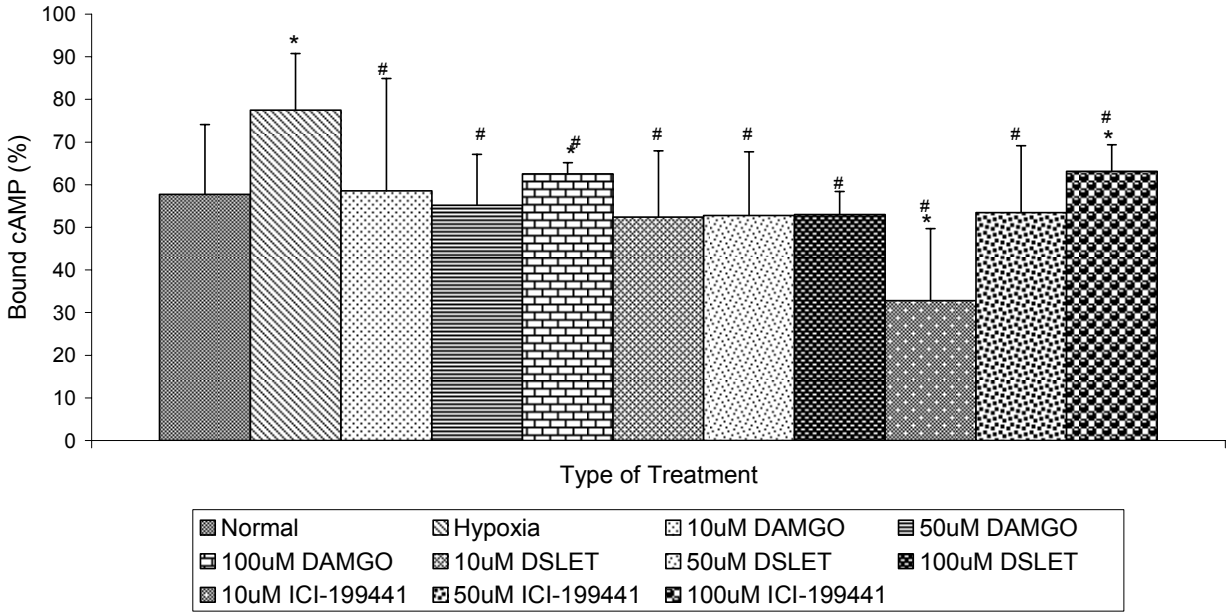


Fig.3.43. The effect of opioid agaonist treatment on bound intracellular cAMP levels in B50 cells in hypoxia.



The effect of opioid agonist treatment on intracellular and bound cAMP levels in B50 cells in hypoxia, as estimated by cAMP immunoassay. Cells were cultured for 48hrs in hypoxia ($5\%O_2;5\%CO_2$) and then treated for 48hrs with different concentrations of different opioid agonists for a total of 96hrs culture. The absorbance of the cAMP assay (n=6) was measured at a wavelength of 410nm. The intracellular cAMP from the untreated normal ($21\%O_2$; $5\%CO_2$) cultured B50 cells was used as the control (Data as mean ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus hypoxia untreated; Student's t-test).

3.10 The effect of hypoxia on extracellular signal regulated receptor kinase (ERK) in B50 neuronal cells in culture.

2.10.1 Quantitative determination of the effect of hypoxia on Phospho-ERK1& 2 in hypoxic treated B50 cells with cannabinoid agonists.

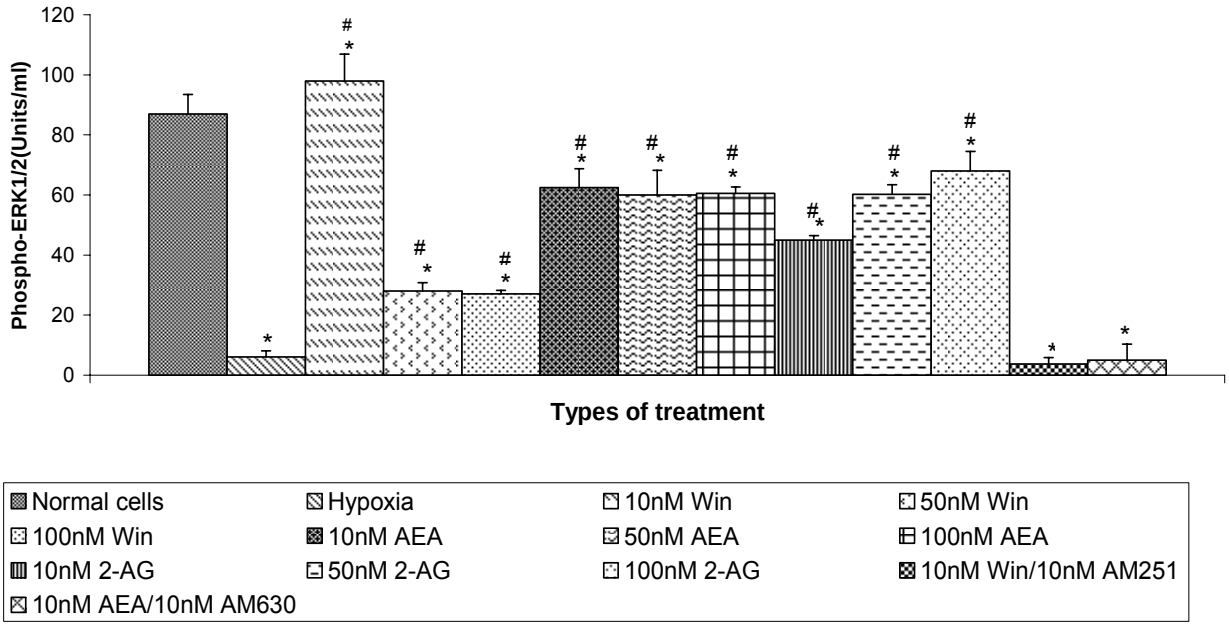
The quantitative determination of the effect of hypoxia on phospho-ERK1& 2 in untreated hypoxic cells and hypoxic cells treated with different concentrations of cannabinoid receptor agonists is shown in Figure 3.44. The results showed that the normal cultured B50 cells had 87.0units/ml of phospho-ERK1/2 which was significantly decreased ($p<0.05$) in untreated hypoxic cells to 6.0units/ml (Table 3.20). The result also showed a significant increase ($p<0.05$) in phospho-ERK1/2 in B50 cells in all cannabinoid treated hypoxic B50 cells when compared to the hypoxic untreated B50 cells (6.0units/ml). However in cells treated with cannabinoid agonist and antagonist, there was no significant difference between them and untreated hypoxic cells (Table 3.20).

Table 3.20: Quantitative determination of the effect of hypoxia on phospho-ERK1&2 levels in B50 cells treated with cannabinoid agonists and antagonists.

Type of Treatment	Net Optical Density (NoD)(450nm)	PhosphoERK1/2 (Units/ml)
Normal cells	2.59±0.65	87.0
Hypoxia no Drug	0.35±0.21	6.0*
Hypoxia 10nM Win	2.81±0.89	98.0*#
Hypoxia 50nM Win	1.02±0.28	28.0*#
Hypoxia 100nM Win.	0.97±0.12	27.0*#
Hypoxia 10nM AEA	2.02±0.62	62.0*#
Hypoxia 50nM AEA	1.90±0.82	60.0*#
Hypoxia 100nM AEA	1.97±0.22	60.5*#
Hypoxia 10nM 2-AG	1.59±0.15	45.0*#
Hypoxia 50nM 2-AG	2.05±0.32	60.25*#
Hypoxia 100nM 2-AG	2.39±0.65	68.0*#
Hypoxia 10nM Win/10nM AM251	0.29±0.20	3.75*
Hypoxia 10nM AEA/10nM AM630	0.34±0.53	5.0*

The effect of hypoxia on phospho-ERK1&2 levels in B50 cells treated with cannabinoid agonists. Cells were cultured for 48hrs in hypoxia ($5\%O_2;5\%CO_2$) and then treated for 48hrs with different concentrations of cannabinoid agonists for a total of 96hrs of culture. The absorbance of the ERK1&2 assay (n=6) was measured at a wavelength of 450nm.Phospho-ERK1&2 from the untreated normal ($21\%O_2;5\%CO_2$) .B50 cells was used as the control. (Data presented as mean ±SD; *$P<0.05$ versus untreated normal cells; #$P<0.05$ versus hypoxia untreated; Student's t-test).

Fig.3.44.The effect of cannabinoid agonist on phospho-ERK1/2 levels in B50 cells in hypoxia as estimated by direct immunoassay

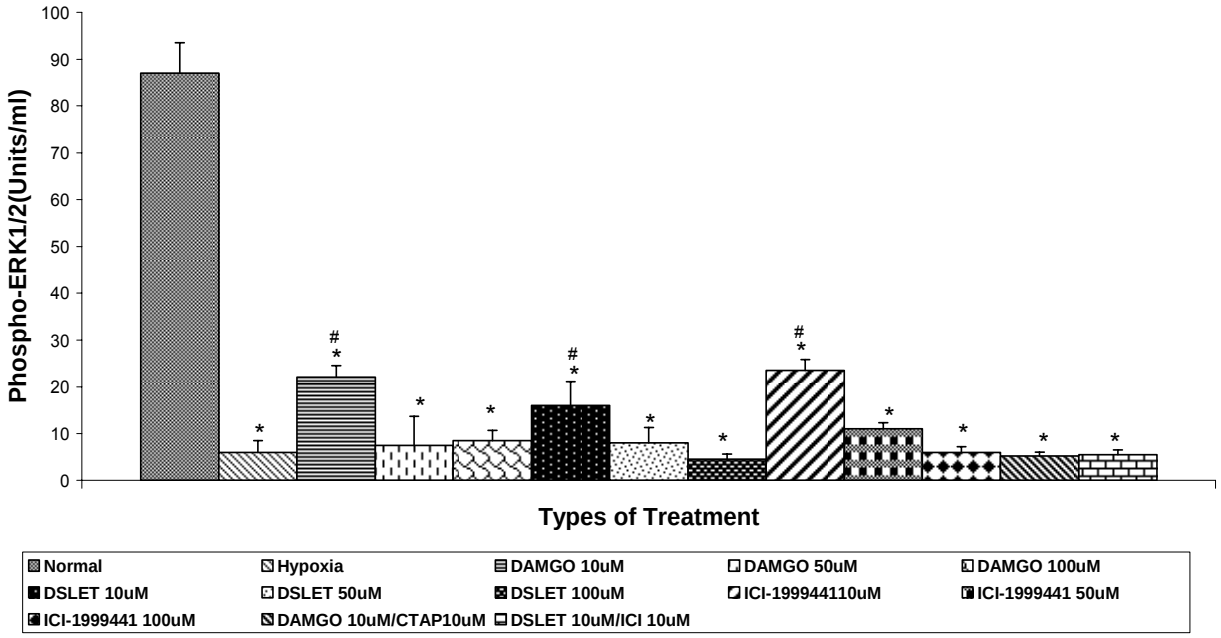


The effect of cannabinoid agonist on phospho-ERK1&2 levels in B50 cells in hypoxia. B50 cells were cultured for 48hrs in hypoxia ($5\%O_2$;$5\%CO_2$) and then treated for 48hrs with different concentrations of cannabinoid agonists for a total of 96hrs of culture. The absorbance of the phospo-ERK1/2 assay (n=6) was measured at a wavelength of 450nm. Phospho-ERK1&2 from the untreated normal ($21\%O_2$; $5\%CO_2$) B50 cells was used as the control (Data presented as mean ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus hypoxia untreated; Student's t-test).

2.10.2. Quantitative determination of the effect of hypoxia on Phospho-ERK1 & 2 in B50 cells treated with opioid agonists.

The quantitative determination of the effect of hypoxia on phospho-ERK1&2 in hypoxic B50 cells treated with opioid receptor agonists is shown in Figure 3.45. There was a significant decrease ($P<0.05$) in the quantity of phospho-ERK1&2 in hypoxic untreated B50 cells (6units/ml), compared to the normal cultured B50 cells (87units/ml). There was also a decrease in phospho-ERK1&2 in B50 cells treated with opioid agonists as shown in Table 3.20. When the quantity of phospho-ERK1&2 in the hypoxic untreated B50 cells was compared to the cells with different concentrations of opioid agonist treatments, the result showed a significant ($P<0.05$) increase in cells treated with 10μM DAMGO (22.0units/ml); 10μM DSLET (16units/ml) and 10μM ICI-199441 (23.5units/ml) (Table 3.21).

Fig.3.45.The effect of opioid agonist on Phospho-ERK1/2 levels in B50 cells in hypoxia as estimated by direct immunoassay



B50 cells were cultured for 48hrs in hypoxia ($5\%O_2;5\%CO_2$), then treated for 48hrs with different concentrations of opioid agonists for a total of 96hrs of culture. The absorbance of the phospho-ERK1/2 assay (n=6) was measured at a wavelength of 450nm. The phospho-ERK1&2 from the untreated normal ($21\%O_2;5\%CO_2$) B50 cells was used as the control (Data as mean ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus hypoxia untreated; Student's t-test).

Table 3.21: Quantitative determination of phospho-ERK1&2 levels in B50 cells treated with opioid agonists

Type of Treatment	Net Optical Density (NoD)(450nm)	PhospoERK1/2 (Units/ml)
Normal Cells	2.59±0.65	87.00
Hypoxia no Drug	0.35±0.21	6.00*
Hypoxia 10µM DAMGO	0.80±0.25	22.0*#
Hypoxia/50µM DAMGO	0.40±0.62	7.50*
Hypoxia100µM DAMGO	0.44±0.22	8.50*
Hypoxia/10µM DSLET	0.65±0.51	16.00*#
Hypoxia/50µM DSLET	0.42±0.33	8.00*
Hypoxia/100µM DSLET	0.31±0.11	4.50*
Hypoxia/10µM ICI-199441	0.88±0.23	23.50*#
Hypoxia/50µM ICI-199441	0.52±0.13	11.00*
Hypoxia/100µM ICI-199441	0.34±0.12	6.00*
Hypoxia/10µM DAMGO/10µM CTAP	0.33±0.08	5.25*
Hypoxia/10µM DSLET/10µM ICI-174864	0.34±0.10	5.50*

Quantitative phospho-ERK1&2 levels in B50 cells treated with opioid agonists in hypoxia as estimated by direct phospho-ERK1/2 immunoassay. The B50 cells were cultured for 48hrs in hypoxia (5%O_2;5%CO_2), then treated for 48hrs with different concentrations of opioid agonists for a total of 96hrs of culture. The absorbance of the phospho-ERK1&2 assay (n=6) was measured at a wavelength of 450nm. Phospho-ERK1&2 from the untreated normal (21%O_2;5% CO_2) B50 cells was used as the control (Data presented as mean ±SD; *$P<0.05$ versus untreated normal cells; $^{\#}P<0.05$ versus hypoxia untreated; Student's t-test).

CHAPTER FOUR

4. Discussion

4.1 General

The B50 neuronal cells used in this study were able to grow, proliferate and differentiate well in culture and were shown to be a very good model of adherent cells since they could attach themselves and grow very well in culture plates. They were shown to be a good experimental model for research in neuronal morphology and cell biology in culture as they were able to grow to confluency levels of about 90-95 % in culture (Table 3.1 and Plates 2 and 4). This is in agreement with Otey et al. (2003), who also showed that the B50 cells could grow, proliferate and differentiate well in culture. The B50 cells, showed a remarkable neuronal pattern in culture resembling very closely those patterns seen in neuronal cells *in vivo* (Plates 3, 4 and 5). These patterns could result in the formation of nerve fibre bundles that form the neural tracks and networks needed for normal and accurate neuronal signal generation, transduction, coordination, relay and interpretation that normally occur in the mammalian nervous system. The cells are easy to handle and maintain in the laboratory environment, provided that their nutritional and general needs are met, and the general aseptic conditions of the laboratory, the culture incubators and the Laminar flow fume chamber are maintained to the highest standard. The cells have also been shown to be a useful model in drug testing since the effects of the drugs on B50 cells can easily be visualised morphologically (Plates 6, 7 and 8).

The result of the effect of hypoxia on neuronal morphology, showed masses of dead and degenerating neuronal cells as shown in Plates 6, 7 and 8. These groups of dead and degenerating cells could result in loss of neuronal mass and volume *in vivo*. This is in agreement with reduced neuronal mass and volume as seen in cases of neurodegenerative diseases like stroke, dementias, Alzheimer's Disease and Parkinson's Disease. However, when these neuronal losses are applied to the whole brain, the overall effect will depend on the part of the brain tissue that the cell loss occurred, which would determine the neurodegenerative disease outcome.

Rodrigo et al. (2005) have shown that a dramatic reduction of oxygen supply to the brain may provoke ischaemia in the whole brain (global ischaemia) or in defined cerebral territories (focal ischaemia). The effect of ischaemic/hypoxic damage to the brain varies according to the area of the brain affected. It has been shown that ischaemia results in severe focal and global damage of the brain tissues accompanied by biochemical and molecular alterations, while hypoxia results in the depletion of cellular and tissue energy and consequently the death of the cells involved (Rodrigo et al., 2005; Szatkowski and Attwell, 1994).

The present results show that there were morphological changes in the B50 cells in hypoxia (Plates 6, 7 and 8), when compared with those B50 cells in hypoxia either treated or pre-treated with cannabinoid and opioid receptor agonists (Plates 10, 11,15, 27, 28 and 29). This shows that the cannabinoid and opioid agonists could have some potential protective and therapeutic benefits in hypoxic conditions by rendering B50 cells treated with cannabinoid and opioid receptor agonists healthier morphologically when compared with the untreated hypoxic B50 cells in culture. The B50 cells treated with cannabinoid and opioid agonists showed more robust morphology when compared to the untreated hypoxic B50 cells in culture as evidenced from their appearances under the microscope. Zhang et al. (2006), have shown that prolonged hypoxia may impair neuronal activities and functions, and thus causes neuronal injury. The activation of the opioid and cannabinoid receptors have been shown to protect cortical neurons from neuronal stress, hypoxia or glutamate induced injury, whereas the antagonism of the receptors, blocks such action (Zhang et al., 2002; Ugdyzhekova et al., 2002; van der Stelt et al., 2001). The present results have demonstrated the presence of cannabinoid (CB_1) and opioid receptors in B50 cells of which their activation by the agonists could have resulted in the observed effects in B50 cells cultured in hypoxia.

Two mechanisms can be proposed for the death and degeneration of B50 cells in hypoxia. It could be as a result of increased intracellular calcium ions and the generation of reactive oxygen species in the cells. The increase in intracellular calcium ions results in excitotoxicity leading to neuronal cell death (Bickler and Buck, 1998). When B50 cells are exposed to hypoxia, the hypoxia may cause an increased excitation of the cells leading to the opening of the ionotropic channels. The

opening of these ionic channels may facilitate calcium ions entry into the B50 cells and the greater the accumulation of the calcium ions, the more excited the cells become. It has been shown that in hypoxia, there is an increase in extracellular excitatory neurotransmitters and Ca^{2+} which causes excitotoxicity resulting in neuronal cell death. The activation of cannabinoid and opioid receptors may play a role in the feedback regulation induced by hyperpolarization and lowering of neuronal excitability by lowering the calcium ion entry into the cells, and consequently lowering the excitotoxic effects of hypoxia on the cells (Zhang et al., 2006; Buzas et al., 1998).

The second mechanism of hypoxia induced cell death is the formation of reactive oxygen species (ROS) within the B50 cells. The ROS that could be generated during hypoxia include oxygen ions, free radicals and peroxides. The ROS are small molecules which are highly reactive due to the presence of unpaired electrons. These ROS are formed as a natural by-product of the normal metabolism of oxygen and have important roles in cell signalling. However, during times of cellular environmental stress such as hypoxia, resulting in oxidative stress, ROS levels can increase and may be responsible for damage to cell structures. This cellular damage resulting in death and degeneration of B50 cells could be as a consequence of apoptosis or programmed cell death and ischaemic injury. This is the situation seen in some neurodegenerative disorders such as stroke, dementias and heart attack. This ROS could result in damage to DNA, oxidation of polyunsaturated fatty acids in neuronal membrane lipids and oxidation of amino acids in the proteins of neuronal membranes (Bickler and Buck, 1998; Trushina and McMurray, 2007), which results in cellular death and degeneration as shown in the case of B50 cells in hypoxia.

The action of opioid agonists could have resulted in the lowering of Ca^{2+} entry into the neuronal cells leading to the opioid receptor upstream regulation as an early response of neurons to hypoxia. Thus it is possible that the increase in opioid receptor density as shown by Gross (2003) and Buzas et al. (1998), was partially attributable to an increase in intracellular Ca^{2+} concentration through Ca^{2+} channels activated by channel depolarization. The result of the morphological changes as shown in Plates 27-29 suggest that opioid receptors are important mediators of the protective response. Buzas et al. (1998) and Gross, (2003), have shown that opioid receptors

participate in ischaemic preconditioning-induced myocardial protection and as such may serve as a general protector against ischaemic/hypoxic stress in oxygen/energy-sensitive organs like the brain and heart (Zhang et al., 2006; Peart et al., 2005; Berridge et al., 2000). The morphological changes presented in the result showed that cannabinoid and opioid receptor agonists may have some potential protective and therapeutic benefits in the treatment of hypoxia in neuronal B50 cells in culture and in other neurodegenerative diseases.

An illustration of the mode of cell death through excitotoxicity caused by Ca^{2+} influx and accumulation of intracellular Ca^{2+} in hypoxic neuronal cell death is shown in Figure 4.1.

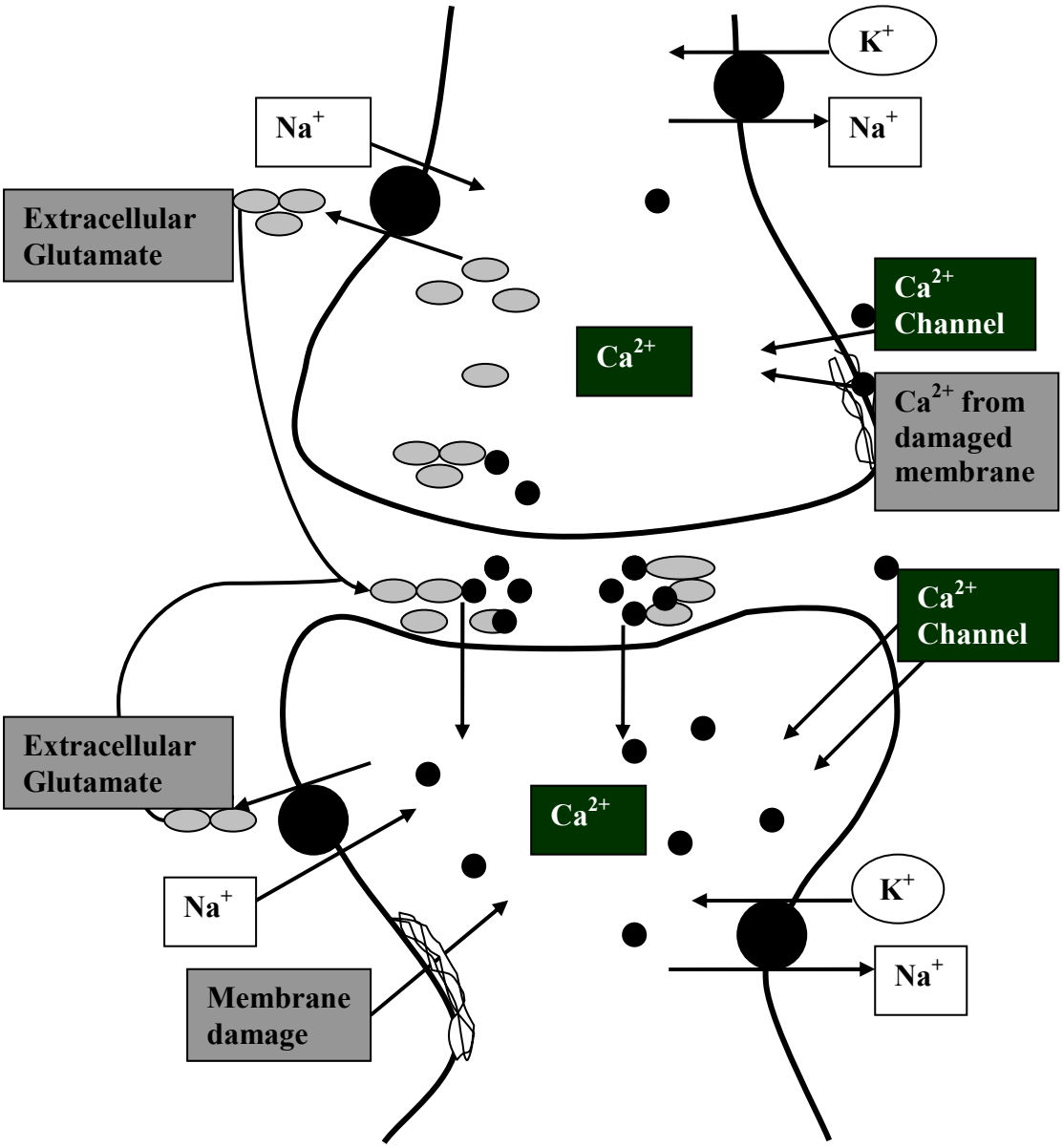


Fig 4.1. Model of cell death through excitotoxicity caused by Ca^{2+} influx (●) and accumulation of intracellular Ca^{2+} in hypoxic neuronal cell death. During hypoxic stress neuronal cell membrane become depolarized triggering Ca^{2+} influx through the Ca^{2+}channels, which results in Na^{+} influx and reversal of Na^{+} gradient and the flooding of extracellular space with excitatory neurotransmitters (⬭), causing cell damage. Adapted from Bickler and Buck (1998).

4.2 Hypoxia

The results presented in Plates 6, 7 and 8; Table 3.2 and Figure 3.4, showed hypoxia-induced changes in the neuronal B50 cell morphology, pattern, proliferation and differentiation. The results showed that hypoxia has an effect on neuronal pattern and pattern forming abilities of B50 neuronal cells when compared to normal B50 cells in culture (Plates 3, 4 and 5). The neuronal pattern is very important for an organised and integrated neuronal activity which could be disrupted if the pattern is disorganised or disoriented (Plates 6, 7 and 8). This is because the normal neuronal organisation involves the grouping of nerve fibres to form nerve bundles of which functionally related nerve bundles ultimately become grouped together and form specific nerve tracts. These linkages of neurons are possible through synapses where signals are passed between cells. The disruption of the neuronal patterns (as shown during hypoxia in this study), indicate that hypoxia could result in the alteration of signal transmission between neuronal cells since some of the neuronal cells are dead and degenerating. These changes in the pattern could also explain the changes in the morphology since the neuronal pattern of arrangement is a part of neuronal morphology.

The effect of hypoxia on B50 neuronal cell proliferation (Table 3.2), showed a time-course effect of hypoxia on B50 cell proliferation in which at 24 and 48 hours of culture, there was no significant change in the cellular proliferation between the normal and hypoxic B50 cells. However, cellular proliferation increased from 72 and 96 hours of culture and then gradually decreased at 120 and 144 hours of culture. The reason the effect of hypoxia on proliferation was not very different at 24 and 48 hours could be as a result of the B50 cells settling down and gradually attaching themselves on the culture plates, then starting to grow and proliferate with the effect of hypoxia becoming pronounced from 72 hours of culture. The results shown in Figure 3.3, indicate that the cellular proliferation in B50 cells increased gradually from 24 hours, reaching a peak at 72 hours and gradually decreasing thereafter. The results suggest that hypoxia may be responsible for the decrease in B50 neuronal cell proliferation when compared with the normal cell in culture.

The result of the effect of hypoxia on cellular differentiation, showed that a higher number of normal B50 cells were differentiated compared to those cultured in

hypoxia only. Hypoxia induced the inhibition of neuronal differentiation by preventing more neurons from differentiating into proper functional neuronal components as possible. The DbcAMP functions by arresting cell division and inducing neuronal differentiation by extension of the neurites (the axon and dendrites), while those cells cultured in hypoxia have more undifferentiated and fewer differentiated B50 cells in culture. However, the effect was more pronounced in those B50 cells cultured initially for 48 hours in normal culture before transferring them to the hypoxic condition for another 48 hours (Figure 3.4). This effect could be due to the shock of transferring these cells suddenly from the normal culture environment ($21\%O_2;5\%CO_2$), to hypoxia ($5\%O_2$; $5\%CO_2$) which could have resulted in more cellular death when compared to the normal and hypoxic groups. This may be due to the adaptation of B50 cells grown exclusively in hypoxic environment.

The changes in morphology, neuronal pattern, proliferation and differentiation that occur during hypoxia in B50 cells in culture were in agreement with the study by Titus et al. (2007), which showed that simulated hypobaric hypoxia, resembling that found in high altitude hypoxia, severely affects the morphology of the central nervous system (CNS) and results in several physiological changes. They showed that these effects specifically within the hippocampus may closely be associated with learning and memory and proposed that insult to this region also affects cognition.

The B50 neuronal cell degeneration in hypoxic cultures may be linked to the loss of neuronal cells and tissues which invariably would result in defects in neuronal activities. These B50 neuronal cell losses and loss of normal neuronal pattern results in loss of neuronal mass, which could lead to loss of nervous system coordination of activities and actions from the cellular level to the whole organism. The neuronal loss may affect cognition, behavioural activities, learning, memory and may even lead to physical defect depending on the area of the brain affected by the degeneration. Studies by Shukitt-Hale et al. (1994), had suggested that rapid or prolonged exposure to hypobaric hypoxia (HBH), is associated with psychomotor and cognitive impairments.

The results presented in this study which showed changes in the characteristics of neuronal B50 cells in hypoxia compared to the normal B50 cells support the work of

Titus et al. (2007), and Shukitt-Hale et al. (1996). They showed that there were structural changes in the neuronal cells of both human and animal models exposed to hypoxia. These structural and morphological changes can lead to defects in memory, cognitive behaviour and loss of coordinated responses to stimuli.

The result with the B50 neuronal cells showed that cell damage increases with the increase in the exposure time to hypoxia (Plates 23 and 29). Thus, the more time the cells are exposed to the hypoxic environment the greater the damage to the B50 cells. This is in agreement with the findings of Titus et al. (2007), which showed that the damage to cells increases with the level of exposure to an oxygen-deprived environment and that the number of damaged cells increases following increase in altitude. Clausen et al. (2005), correlated the hippocampal morphological changes and memory dysfunction, cortical lesion volume and regional hippocampal morphological changes after controlled cortical contusion (CCC) injury in rats, and showed morphological changes in CA1, CA3, and hilus of the dentate gyrus in severely injured rats.

Sousa et al. (2000) have demonstrated that stress-induced cognitive deficits in rats do not correlate with hippocampal neuronal loss when they evaluated the effects of chronic stress on hippocampal dendrite morphology, volume of mossy fibre system, and number and morphology of synapses between the mossy fibres. They found profound changes in the morphology of the mossy fibre terminals and significant loss of synapses were detected in stress-induced cognitive conditions (Sousa et al., 2000). The study by Sousa et al. (2000), showed that stress-induced morphological changes in the hippocampus could not be correlated with cognitive changes in rat.

The data presented here showed that hypoxia had an effect on B50 neuronal cell morphology, pattern, proliferation and differentiation which could have a deleterious effects on neuronal structure and morphology which would result in overall functional impairment of the nervous system in terms physical activities, cognitive abilities, learning and memory functions even though this may depend on the parts of the brain that are affected.

4.2.1 Hypoxia tolerance

The result presented in Plates 6-9, showed that some of the B50 cells in hypoxia did not degenerate and had normal structural features as evidenced from their morphology. This is an indication that different cells within the same environment have different ways of reacting to the same conditions. The result from investigation of B50 cells cultured under hypoxia showed that some B50 cells can react to hypoxic conditions differently, while some would die and degenerate others may survive for a longer period of time even when other cells within the same environment have died and degenerated (Plates 6 and 7). This situation may be as a result of these neuronal B50 cells that survived being hypoxia tolerant. Lipton (1999) and Vornov et al. (1998), have shown that distinct neuronal populations have very different vulnerabilities to ischaemia and showed that at 5-minutes, global ischaemia caused delayed death in almost all CA1 pyramidal cell neurons with no effects on other populations, whereas at 20 minutes, ischaemia caused cell death in CA3 neurons but had almost no effect on dentate granule cells in the hippocampus or on interneurons in CA1 (Vornov et al., 1998). Wenger, (2002), observed that some cells were able to adapt to hypoxia by the use of oxygen sensing proteins such as hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression.

In addition, Lipton (1999) have shown that the differences in presynaptic events may be responsible for apparent selective vulnerabilities of postsynaptic neurons. These large differences in selective vulnerabilities between neuronal cells are indicative of large differences in metabolism between different B50 cell types that presumably reflect their different physiological functions which may lead to differential vulnerabilities to ischaemia and hypoxia which may provide insight into mechanisms of cellular damage during hypoxia in B50 cells in culture.

The reason why some of the B50 cells did not degenerate during hypoxia could be because hypoxia decreases metabolic demands and energy production of these B50 cells, which may potentially lead to tolerance of these B50 cells to hypoxia. This hypothesis suggests that energy conservation leads to metabolic and ion channel arrests which must have accompanied decreased energy production to maintain energy charge, substrate stores, and ionic homeostasis during hypoxia in B50 cells thus leading to some of the cells surviving during the hypoxic condition in the culture

environment. With respect to ion homeostasis, the B50 cells that survived prevented the collapse of transcellular ion gradients, and may generally decrease the activity of ion pumps more than ion leaks. Ion channels responsible for ionic leakage, potassium channels, became relatively insensitive to the hypoxic changes in the B50 cells, whereas ion-motive pumps such as the Na^+/K^+ pump are more strongly inhibited by temperature changes in some cases (Boutilier, 2001). The disparity between ion pumping and ion channel leakage rates leads to a redistribution of ions, depolarization of transcellular electrical potentials, and water accumulation in hypoxia-intolerant B50 cells, leading to the death and degeneration of these B50 cells while the hypoxia-tolerant would survive in such adverse conditions (Boutilier, 2001).

It has been shown that oxygen depletion is relatively common in some environments and represents both a challenge and an opportunity for organisms that survive there (Buck and Pamenter, 2006). A commonly observed survival strategy to this kind of stress environment by cells is to lower metabolic rate known as metabolic depression. Whether metabolic rate is at a normal or a depressed level, the most important thing is that the supply of ATP from glycolysis and oxidative phosphorylation must match the cellular demand for ATP for protein synthesis and ion pumping, a condition that must be met for long-term survival in hypoxic and anoxic environments (Buck and Pamenter, 2006).

The above could be the situation for the survival of some of the B50 cells shown in the hypoxic condition as presented (Plates 6 and 7), while other B50 cells died and degenerated. The survival of some B50 cells could actively be regulated by second messenger pathways but it is less clear if they are regulated differentially or sequentially with the onset of hypoxia. The vertebrate brain is extremely sensitive to low oxygen levels yet some species survive in oxygen depleted environments for extended periods. Hypoxia/ischaemia tolerance mechanisms in liver and heart have been shown to offer clues to how the brain can tolerate anoxia (Buck and Pamenter, 2006). Furthermore, it has been shown that mitochondria are ideally situated to serve as cellular oxygen sensors and mediator of protective mechanisms such as ion channel arrest (Buck and Pamenter 2006).

Hypoxia has been shown to kill neurons because anaerobic energy production could not keep pace with demands (Bickler and Donohoe, 2002). Many types of hypoxia-

tolerant neurons solve this risk of energy failure not by increasing anaerobic metabolism, but by decreasing the demand for energy sufficiently to prevent the loss of high-energy metabolic intermediates such as ATP and phosphocreatine (Hochachka ,1986). When oxygen flow to the mammalian brain decreases to critical levels, energy failure occurs, with a decline in ATP and when half of the ATP is lost, depolarization of the membrane and subsequent uptake of sodium and water occurs (Knickerbocker and Lutz, 2001; Erecinska et al., 2005). Depolarization causes Ca^{2+} influx through voltage-gated Ca^{2+} channels. The Na^{+} gradient collapse causes the sodium-glutamate co-transporters to eject glutamate into the extracellular space (Rossi et al., 2000). Glutamate triggers vigorous activation of glutamate receptors, initiating a process of calcium influx and excitatory injury. Glutamate receptors have been primary targets for experimental treatment of ischaemic brain injury (Choi, 1995; Lee et al., 1999). The death of neurons from these insults could follow quickly from swelling and lysis (necrosis) or may be gradual over many days. This slower cell death is complex and, in some respects, resembles programmed cell death or apoptosis.

A most fundamental adaptive response of hypoxia-tolerant cells to oxygen lack is the capacity to avoid a drastic decline in ATP levels at a time of absent aerobic ATP production. It is likely that similar responses to energetic stress are found in cells during hibernation and aestivation, although hypoxia does not characterize these states. A drastic, balanced, suppression of ATP demand and supply pathways must occur in all these conditions, this regulation allows ATP levels to remain relatively constant, even while ATP turnover rates greatly decline. In neurons, the ATP requirements of ion pumping (mainly Na^{+}) are down regulated by channel arrest (Bickler et al., 2002). The ATP demands of protein synthesis also must be down regulated, and although rapid and global suppression of protein synthesis occurs in anoxia-adapted hepatocytes (Hochachka et al., 1996), Acker and Acker (2004) showed that it is likely that selective rather than global suppression of gene expression and protein synthesis occurs in neurons. This necessitates that a significant percentage of the neurons in the brain are functionally inactivated during anoxia, therefore cannot participate in vigilance or regulatory activities. Accordingly, metabolic arrest cannot be the only cellular response for vertebrate neurons, some neuron groups must remain vigilant and participate in regulation and eventual arousal from dormancy or inactivity

(Milton et al., 2007). Cellular adaptations essential for survival during hypoxia are illustrated in Figure 4.2.

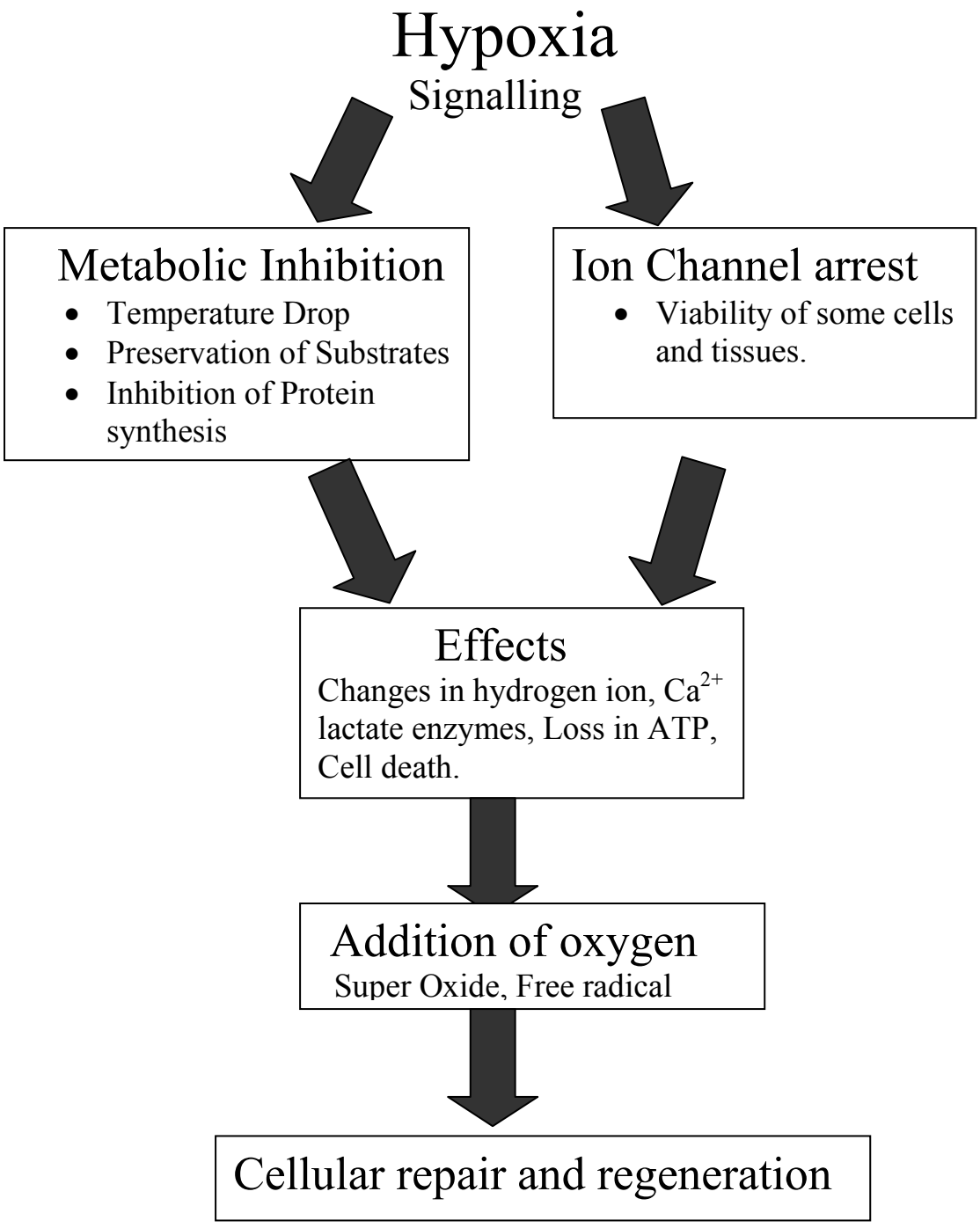


Fig.4.2. Adaptations essential for surviving during hypoxia in cells depending on the species and tissues involved. Adapted from Bickler and Buck, (2007).

4.3 Morphological changes

Hypoxia has been shown to result in depletion of cellular and tissue energy which consequently results in death of the cells and tissues involved. It is probable that many of the B50 cells in hypoxia underwent degeneration due to depletion of cellular energy which could have resulted in the death of these cells during hypoxia. It has been shown that oxygen delivery to the brain cells and tissues depends on the volume flow rate of blood and arterial oxygen content of which a reduction in any of these two parameters gravely affects vital brain functions (Rodrigo et al., 2005). In hypoxia and ischaemia, there is the tendency of production and accumulation of toxic substances, most especially carbon monoxide with concomitant deterioration and damage to neuronal cells and tissues (Bickler and Donohoe, 2002; Rodrigo et al., 2005).

Cerebral hypoxia/ischaemia results in a cascade of molecular events including rapid depletion of intracellular adenosine triphosphate (ATP) stores, anaerobic glycolysis, lactic acidosis and membrane depolarization, glutamate excitotoxicity, entry of calcium, sodium and water into the cells, resulting in cell swelling, activation of calcium-stimulated enzymes, mitochondrial dysfunction, free radical production, activation of the immune system, over expression of particular genes and consequently increased neuronal death (Agar et al., 2000; Acker and Acker, 2004). Hypoxia has been implicated in central nervous system pathology in a number of disorders including stroke, head trauma, neoplasia and other neurodegenerative diseases.

Hypoxia/ischaemia compromises membrane functions and cellular enzymes like xanthine dehydrogenase which catalyses the reduction of molecular oxygen to form both superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2). Hypoxia/ ischaemia in the brain leads to extensive neuronal damage and subsequent neurological impairment. Some mechanisms could be proposed for the death of the B50 cells during hypoxia which include oxidative stress and glutamate toxicity. Cellular death of the B50 cells by hypoxia/ischaemia could be as a result of apoptosis or direct necrosis induced by oxygen and glucose lack and may also be by cytotoxic agents like carbon monoxide (CO), carbon dioxide (CO_2) and nitric oxide (NO) and proinflammatory cytokines. The role of free radical, NO produced by nitric oxide

synthase (NOS), has been shown to occur in acute cerebral ischaemia and chronic neurodegenerative diseases like schizophrenia, Alzheimer's disease and Parkinson's disease and aging dementia (Giannakopoulos et al., 2003; Mazarakis et al., 2005, Jellinger, 2007).

It has been shown that the neuronal NOS isoform (nNOS) occurs in the brain neurons and generates NO which acts as a neurotransmitter (Rodrigo et al., 2007). Also inducible NOS (iNOS), a Ca^{2+}/calmodulin-independent enzyme, is responsible for a high output of NO and is induced by the exposure to cytokines and lipopolysaccharide, and has been characterised in many cell types as a consequence of the inflammatory processes that follow infection, disease or tissue damage (Rodrigo et al., 2007). The iNOS is expressed by macrophages, neutrophils, vascular smooth muscles, endothelial cells, microglia, astrocytes and neurons of the cortical areas and cerebellum (Rodrigo et al., 2005; Rodrigo et al., 2007). Inducible NOS expression has been shown to form NO in many tissues of the body in response to diverse cell stresses, following injury, opiate dependence, trauma and cerebral ischaemia and several pathologies including demyelinating diseases, AIDS, dementia, amyotrophic lateral sclerosis, Alzheimer's disease and epilepsy (Rodrigo et at., 2005; Yahyavi-Firouz-Abadia et al., 2004).

NO is associated with neuronal cell death (Hewett et al., 1994; Kolker et al., 2001). The adverse effects of NO in eukaryotic cells is related to the mitochondria of which the alteration of mitochondrial function seriously affects cell viability which can lead to necrosis through irreversible mitochondrial damage and collapse of the energetic capacity of the cell (Cramer and Sur, 1999; Andersen, 2004). Alternatively NO can induce apoptosis when proteins released from the mitochondria initiate caspase activation and the formation of a complex with apoptotic protease-activating factor (Weinmann et al., 2004a). The release of cytochrome c from mitochondria during hypoxia has been shown to be controlled by members of the Bcl-2 family which inhibit cell death and prevent the release of cytochrome c while other proteins such as Bax and Bak promote cell death and induce cytochrome c release (Weinmann et al., 2004a Weinmann et al., 2004b). Also the product of the reaction between superoxide O_2^- and NO called peroxynitrite ($ONOO^-$) inhibits mitochondrial respiration and stimulates apoptosis (Rodrigo et al., 2005; Mishra et al., 2006). Xu et al. (2001), have

observed that amyloid beta peptide-induced cerebral endothelial cell death involved mitochondrial dysfunction and caspase activation. Also ischaemia and severe hypoxia results in release of cytochrome c from mitochondria (Zhu and Bunn, 2001). Thus the mitochondrial swelling associated with ischaemia or glutamate excitotoxicity could be due to the mitochondrial permeability transition (MPT) induction by NO which reflects the formation of proteinaceous pores in the mitochondrial membrane that allows free diffusion of small molecules (Mishra et al., 2006; Rodrigo et al., 2005). Figure 4.3, illustrates the effect of oxidative stress in cells, leading to generation of reactive oxygen species and the consequent oxidative damage.

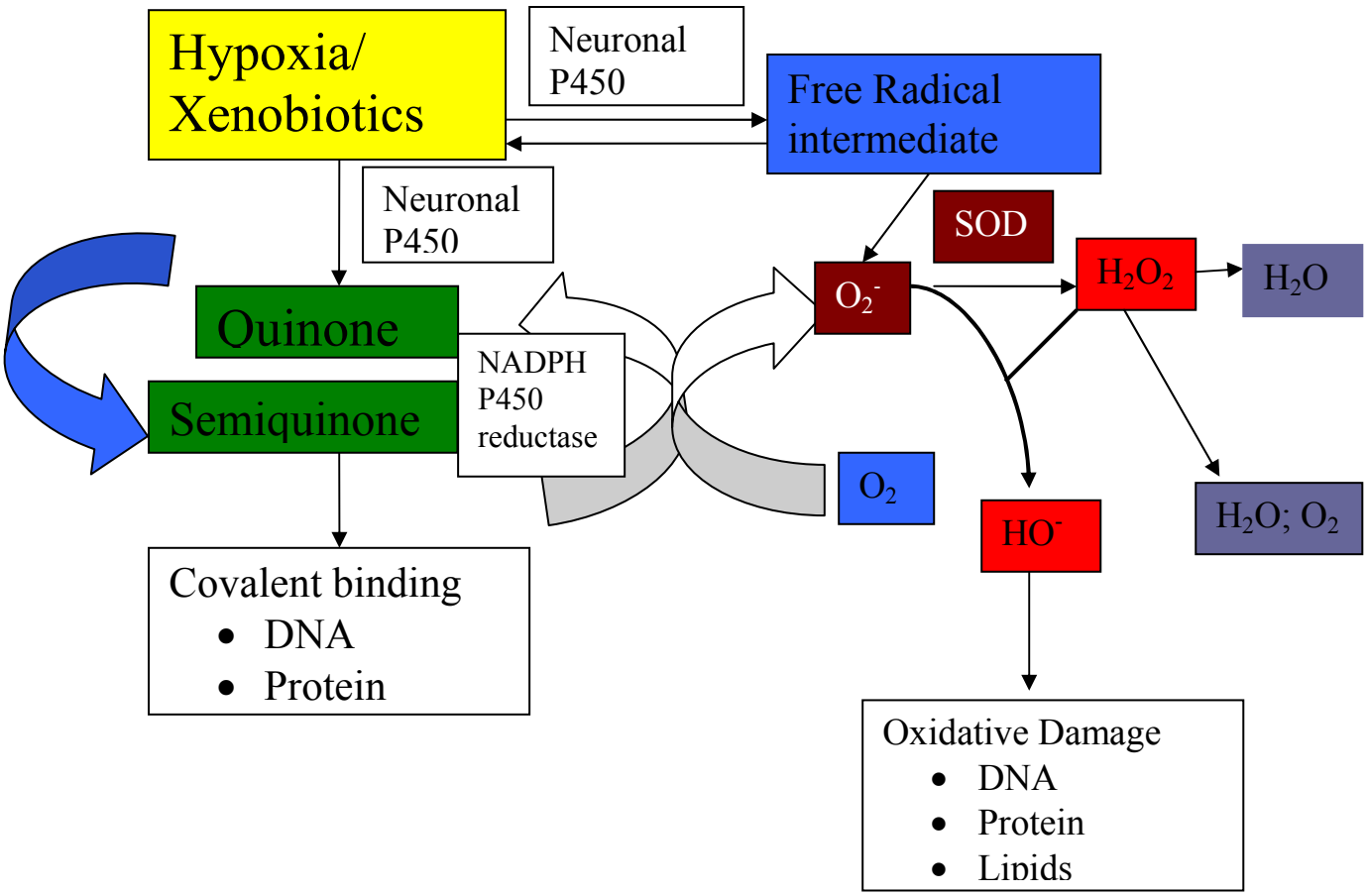


Fig.4.3. Oxidative stress such as hypoxia is imposed on cells as a result of increase in oxidant generation, a decrease in antioxidant protection, or a failure to repair oxidative damage. Cell damage is induced by reactive oxygen species. The main damage to cells results from the ROS-induced alteration of polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. Adapted from Sigma (2006).

4.4. Cell viability

Since the B50 cell line was developed from neonatal neuronal cells, it means that these cells could grow, proliferate, and differentiate into mature neuronal tissues (Plates 1-5). However during development, most developing neural systems undergo a period of heightened neuronal cell death during which about 30-80% of their constituent neurons are lost during the time of their initial contact with innervation targets (Carr et al., 2005; Lobner et al., 2000). Middleton et al. (2001), showed that cranial sensory and parasympathetic ganglia revealed losses of about 30% in the mouse nodose ganglion, 35-55% in the mouse trigeminal ganglion and 50% in the rat vestibular ganglion and chick ciliary ganglion. There is elevated cell death in the geniculate ganglion neuronal population with a degeneration pattern similar to those seen in the vestibular, spinal and trigeminal ganglia (Nikolic et al., 2000; Lobner et al., 2000). However, the elevated cell death of geniculate neurons was not accompanied by a major reduction in the neuronal population which suggested an overlapping occurrence of neuronal death and neuronal generation in different neuronal subpopulations (Nikolic et al., 2000; Kim et al., 2006a; Lobner, 2000).

The results from the proliferation, differentiation and morphological studies indicated that the B50 neuronal cell degeneration showed a pattern of neuronal cell death. The B50 neuronal degenerative activity level was significantly higher in hypoxic cultures without treatment than those in hypoxia but with various drug treatments (Figure 3.32). It was seen from the result of this study that the major degenerative peak was found to be at 144 hours of culture in both normal and hypoxic cultured B50 cells (Tables 3.1 and 3.2). This shows that neuronal degeneration increases as the duration the cells stay in the culture increases. The presence of a greater density of dead and degenerating cells in the various experimental models in untreated hypoxic, hypoxic treated and pre-treated B50 cells revealed areas of heightened cell death which may represent cellular death due to developmental or tissue activities (morphogenetic or histogenetic cell death) (Carr et al., 2005) (Plates 6-9 and 24-26). Similar patterns of early degeneration with a later peak, rather than a sharp onset of degeneration corresponding with the time of target innervations was seen in neurons in the vestibular, spiral and trigeminal ganglia (Nikolic et al., 2000). The overall pattern of degenerating neuronal cell count in this study may reflect temporal aspects of responses of neuronal cells to noxious environmental conditions as in hypoxia within

different neuronal subpopulations as demonstrated by Carr et al. (2005) in trigeminal ganglion with different degenerative patterns occurring in some knockout mice.

The neuronal counts (Figures 3.1 and 3.2), made in the course of this study were not intended to provide an absolute total neuronal cell count, because the numbers obtained clearly underestimated the actual number of dead and degenerating B50 neurons in these experimental models. This is because the B50 neuronal cells are adherent or attached cells of which some of the dead and degenerating B50 cells become detached from the culture plate and float in the culture media. Hence when the media was removed, some of these cells are lost with the media. While the numbers obtained may underestimate the number of dead and degenerating B50 neuronal cells, they provide a reliable indication of the pattern of the B50 neuronal cell population dynamics as evidenced from the results in control groups, and in both treated and untreated hypoxic B50 cells in culture.

The data presented in Plates 3 and 4, and Figures 3.1 and 3.2, shows a striking feature of B50 cells in that they do not show any period of major neuronal loss in the control group when compared with the experimental or hypoxic groups. The control B50 cells showed a continual rise in neuronal cell numbers from 48 hours through 144 hours of culture with only slight decrease between 0 hours and 12 hours of culture in which the cells are trying to settle down to their new environment and start a new life after being disturbed by the harvesting and splitting of the cells. Some of the cells may be lost due to stress from the processes they pass through during splitting and sub-culturing. This is in contrast to the pattern seen in many developing neural systems in which not only are large numbers of neurons lost following initial target contact but this loss occurs over a limited period of time (Carr et al., 2005). Carr et al. (2005), showed that different subpopulations differed slightly in their temporal patterns of precursor proliferation and neuronal cell death and that accretion of neuronal cell numbers via proliferation and differentiation in one population could mask the degree of neuronal loss occurring simultaneously. The generation of free radicals which occurs in most oxidative stress conditions, causes free-radical signalling, defence, and injury which may result in the loss of cell viability and damage, and consequent death in neuronal cells as illustrated in Figure 4.4.

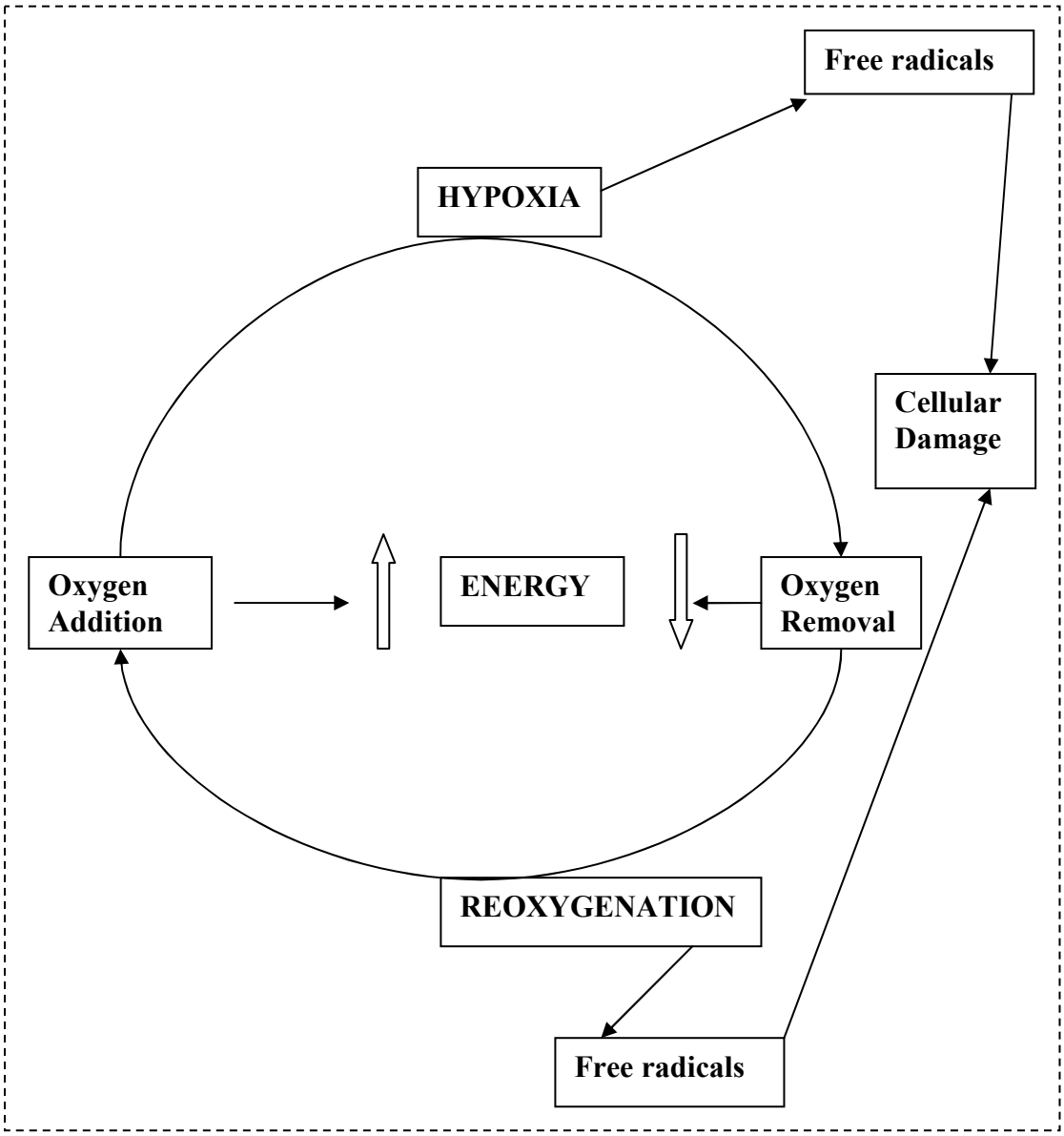


Fig.4.4. Oxidative stress in tissues experiencing cycles of oxygen presence, showing cyclic nature of free-radical generation during oxygen and hypoxia transitions. Adapted from Bickler and Buck (2007).

4.4.1. LDH assay of cell viability

Lactate dehydrogenase (LDH) leakage into the culture supernatant of untreated B50 cells exposed to hypoxia showed more cellular death which may be as a result of the B50 neuronal cell membrane disintegration (Koh and Choi, 1987) (Table 3.3). The neuronal cell membrane disintegration allows the leakage and release of the cytoplasmic bound LDH enzymes into the culture medium, thus causing more neuronal cellular death. This additional cellular death could be corroborated by more groups of dead and degenerating B50 cells in hypoxia (Plates 6, 7, 23 and 26), when compared with the normal B50 cells (Plates 16, 22 and 24) and hypoxic treated and pre-treated B50 cells (Plates 15, 18, 27 and 33). The results from Table 3.3 and Figure 3.5 show a time course comparative LDH release from both the normal and hypoxic B50 cells in culture. The results showed that there was a time course in the leakage of LDH from the B50 cells in both normal and hypoxic environments. The results showed that the length of time the cells stay in culture determines the amount of LDH they released into the media in both normal and hypoxic cells though the cells cultured in hypoxia had a significant increase in the LDH released into the cellular media when compared with those in normal culture.

The question that arises is, if the normal cells were under optimal conditions, why were they releasing or leaking LDH into their media? The release of LDH by the normal B50 cells may be that since the B50 cells are neonatal cells, they are subject to normal developmental processes of which death and regeneration of cells are very common, especially in the neural system in which cells are subjected to continual death and renewal at early stages of their development. This is in agreement with Middleton et al. (2001), who showed that cranial sensory and parasympathetic ganglia losses about 30% of neurons in the mouse nodose ganglion and 35-55% of neurons in the mouse trigeminal ganglion. There was elevated cell death in the geniculate ganglion neuronal population but, the elevated cell death of geniculate neurons was not accompanied by a major reduction in the neuronal population which showed overlapping occurrence of neuronal death and neuronal generation in different neuronal subpopulations (Nikolic et al., 2000; Lobner, 2000). These B50 cellular death and degeneration, resulting in the release of LDH, could also result from either cell necrosis or programmed cell death (apoptosis) that occur as part of the normal cellular developmental processes.

The second reason to account for the LDH leakage from normal cells could be the result of cellular injury and damage during the process of splitting the B50 cells as part of the culturing process. These effects could either be mechanical or chemical in nature. The mechanical injuries and damage could be the result of frictional forces during the shaking and centrifugation process as one of the techniques involved in the splitting of the cells. Since the B50 cells are neuronal in nature, they are highly sensitive and could easily be injured and damaged by such processes leading to LDH leakage from the cell membranes. The chemical injuries and damages could occur due to the use of trypsin-EDTA solution to detach the cells from the base of the culture plate. Since this proteolytic enzyme is an oxidizing agent it could easily injure or damage the membrane resulting in LDH leakage from the cells.

However, LDH release was reduced by cannabinoid and opioid treatment and pre-treatment in B50 cells, which show that these drugs may have potential for protection and therapy in hypoxic conditions. The results presented in Tables 3.9, 3.10, A1, and A2, show the changes in LDH leakage in B50 cells administered with different concentrations of cannabinoid and opioid agonists. The more LDH leaked into the media, the more injurious the cells become and hence the cells are prone to die and degenerate more easily in hypoxic conditions than those in the normoxic conditions and those treated with some of the agonists in hypoxic conditions. The LDH release from the B50 cells could have been as a result of disintegration of the B50 cells due to oxidative stress caused by the hypoxic condition. The results presented in this study showed a concentration-dependent effect of cannabinoid agonists on LDH release from B50 cells. The results showed that the LDH release was increased with increasing concentration of Win agonist in that 10nM Win had LDH release lower that those from the 50nM Win and 100nM Win treated cells in hypoxia, while those treated with AEA and 2-AG had LDH release decreased as the concentration of the agonist increases. Hence the cells treated with 100nM AEA and 100nM 2-AG had LDH release lower than those treated with 50nM AEA, 50nM 2-AG,10nM AEA and 10nM 2-AG. The results showed that different agonist has different trends in LDH release in B50 cells depending on the specific agonists. While Win was better in reducing the LDH release in lower concentration in B50 cells in hypoxia, AEA and 2-AG were most useful at higher concentrations. This is in agreement with the findings of Shivachar (2007), which showed that cannabinoid agonists inhibited sodium-

dependent, high-affinity excitatory amino acid transport in cultured rat cortical astrocytes in concentration-dependent manner.

The results show that the selective cannabinoid (CB_1) antagonist AM251 and the competitive cannabinoid antagonist, AM630 were able to reverse some of the effects of the cannabinoid agonists on LDH release in a concentration-dependent manner. The treatment with 10nM AM251/10nM Win (840%), was able to completely reverse the activity of the agonist 10nM Win (217%) against LDH release in B50 cells in hypoxia (Table 3.11 and Fig.3.16a), while at a higher concentration of both the agonist and the antagonist (50nM Win/50nM AM251; 100nM Win/100nM AM251), the LDH release from the B50 cells in hypoxia was about the same. The LDH release from the treatment with different concentrations of anandamide (AEA) was completely blocked with the equivalent concentrations of the competitive cannabinoid antagonist (AM630) (Table 3.11 and Figure 3.16b). The results also show that the selective cannabinoid (CB_1) antagonist AM251 and the competitive cannabinoid antagonist AM630 were able to completely reverse the effects of the agonists on LDH release in B50 cells pre-treated against hypoxia in culture (Table 3.14 and Figure 3.19). The results showed that the cannabinoid agonist-induced inhibition of LDH release from B50 cells in hypoxia was blocked by the cannabinoid receptor antagonists (AM251 and AM630). The results suggest that the inhibition of LDH release from B50 cells in hypoxia by cannabinoid agonists is mediated through cannabinoid (CB_1) receptors since AM251 and AM630 have been shown to be potent CB_1 antagonists in the brain (Gholizadeh et al., 2007; Werner and Koch, 2003). This is because AM630 though sold as CB_2 antagonist, behaves as a competitive antagonist of Win 55,212-2 and anandemide and as such exerts cannabinoid receptor antagonism in the brain (Pertwee et al., 1995; Hosohata et al., 1997).

The results of the opioid agonist and antagonist treatment on LDH release in B50 cells, showed that the treatment of cells with different concentrations of opioid antagonists were able to reverse the inhibition of LDH release provided by the different opioid agonists in B50 cells in hypoxia. The results suggest that the inhibition of LDH release from B50 cells by opioid agonists is mediated through the opioid (μ, δ and κ) receptors and as such the opioid antagonists act to block the activities of the agonists on the receptors involved, since it has been shown that both

cannabinoids and opioids exert a wide range of their effects through coupling to inhibitory $G_{i/o}$ proteins (Williams et al., 2001). It has also been shown that very low doses of opioids can selectively activate an excitatory G_s protein-coupled signalling pathway (Crain and Shen, 2000), and as such it was suggested that ultra-low doses of an opioid receptor antagonist can block this sensitive excitatory mechanisms and unmask a potent inhibition that is induced by opioids at concentrations lower than their minimal effective doses in some tests such as antinociception (Gholizadeh et al., 2007). Manzanares et al. (1999), have shown that a number of functional interactions exist between cannabinoid and opioid systems which might be a result of shared signal transduction mechanisms (Childers et al., 1992), as well as the release of endogenous opioids by cannabinoid agonists (Valverde et al., 2001).

Mitochondrial dysfunction has been shown to be involved in neuronal damage associated with hypoxic/ischaemic injury, aging and neurodegenerative diseases (Albers and Beal, 2000; Calabrese et al., 2001). Recent studies by Egea et al. (2007), and Weissman et al. (2007), have shown that mitochondrial membrane-potential was decreased during hypoxia/reoxygenation and subsequent Ca^{2+} overload, results in dysfunction of the mitochondria and ultimately cell death, resulting in higher LDH leakage as could be the case of B50 cells in hypoxia when compared with the normal B50 cells in culture. Mitochondrial dysfunction during hypoxia can be related to the depletion of ATP but also to the release of apoptotic proteins and various caspases during hypoxia resulting in cellular deaths (Jayalakshmi et al., 2005; Kreutz et al., 2007). This explains why the hypoxic disturbance of the energy-producing system was likely to be one of the primary signals in hypoxia-induced cell death leading to increased LDH release from the cells in hypoxic cultures as in the case of B50 cells. The B50 cellular death and degeneration could be as a result of stress-induced cellular suicide (Steller, 1995), or some of the cells may undergo a process of dormancy or aestivation which may eventually result in death of these cells (Storey, 2002).

Studies have shown that the increase in intracellular Ca^{2+}-dependent signalling cascades contribute to further free radical formation, energy depletion which eventually leads to neuronal cell death (Choi, 1988; Burgoyne, 2007). Increased intracellular Ca^{2+} levels activate a group of serine proteases called caspases which in-turn activate endonucleases resulting in DNA breaks (Jayalakshmi et al., 2005). The

significant damage to DNA resulting from endogenous free radical attack has already been suggested to contribute to the pathology of cancer and neurodegenerative disease processes (Zhang et al., 1999; Burgoyne, 2007).

The results presented (Tables 3.12, 3.15, A5 and A6), show the LDH release from normal cultured B50 cells administered with different concentrations of cannabinoid and opioid agonists. They show some significant decreases and few insignificant increases in LDH leakage from the treated and pre-treated normal B50 cells when compared to the untreated normal B50 cells. There were decreases in LDH release from the normal B50 cells administered with cannabinoid agonists. The decreases in LDH release could be as a result of these agonists acting as a protective agent to the cells thereby limiting the amount of damage to the cells and hence the quantity of LDH released by these cells into the media. Abood et al. (2001), had shown that the activation of the cannabinoid (CB_1) receptor protects cultured mouse spinal neurons against excitotoxicity induced by kainate. These neuroprotective effects of the cannabinoid agonists were blocked with the administration of CB_1 receptor antagonist, AM251 indicating a receptor-mediated effect. Cannabinoid receptor agonists were neuroprotective in excitotoxic cell death in cultured cerebellar neurons (Skaper et al., 1996). Anandamide had been shown to decrease hippocampal neuronal loss after transient global cerebral ischaemia and reduce infarct volume after permanent focal cerebral ischaemia induced by middle cerebral artery occlusion in rats (Nagayama et al., 1999). Cannabinoid agonists were also shown to be protective in cultured cortical neurons from hypoxia and glucose deprivation though the effect was non-stereo selective and was insensitive to CB_1 and CB_2 receptor antagonists. In this way cannabinoids may have therapeutic potential in disorders resulting from cerebral ischaemia, including stroke, and may protect neurons from injury through a variety of mechanisms (Nagayama et al., 1999; Abood et al., 2001). Cannabinoid receptor agonists have been found to possess antioxidant properties, which may contribute to the reduction of excitotoxicity in neuronal cultures (Eshhar et al., 1995; Hampson et al., 1998).

Some of the cells administered with opioid agonists showed increased levels of LDH release as in those normal cells pre-treated with 50µM DAMGO and 100µM DAMGO and treated B50 cells with 50µM DAMGO, 100µM DAMGO and 10µM

ICI-199441. These increases in LDH release in the treated and pre-treated normal B50 cells could be as a result of concentration-dependent toxicity of that particular drug thereby leading to damage to some of the cells administered with these drugs in the normal condition resulting in the release of higher LDH levels more than those untreated normal cells in culture. Though the opioid receptors have many similarities, one possible explanation for the differences in their neuroprotective capabilities is that the individual opioid receptors regulate different effectors, thereby eliciting different responses (Zhang et al., 2002), and the selectivity of these receptors for eliciting specific pathways does not lie in differences between each opioid receptor subtype but in their association with divergent types of G proteins (Connor and Christie, 1999). The μ, δ and κ-opioid receptors are all capable of interacting with the pertussis toxin-sensitive G-protein α-subunits such as G_{i1}, G_{i2}, G_{i3}, G_{o1}, G_{o2} and the pertussis toxin-insensitive G_z and G_{16} (Connor and Christie, 1999). It has also been shown that each opioid receptor subtype preferentially couples to specific G proteins, example, the delta opioid receptor (DOR) is more efficiently coupled to the $G_{\alpha 16}$ protein than either mu or kappa-opioid receptors (Lee et al., 1998), which may apparently yield specialized differences between the opioid receptor subtypes and the regulation of the effectors. Zhang et al. (2002), had shown that the modulation of DOR plays a major role in neuroprotection in both normoxic and hypoxic environments even though the mechanisms of this action is not yet known, it could be speculated that this phenomenon may be linked to the role of DOR in selective regulation of G proteins, excitatory neurotransmitter release, glutamate receptor stimulation, and Ca^{2+} homeostasis (Howlett et al., 2004; Abood et al., 2001).

The cellular damage that occurs in hypoxia leading to LDH leakage could result due to loss of ion gradient in the B50 cells. This is because neuronal function is dependent on maintenance of Na^+, K^+, Cl^- and Ca^{2+} transmembrane distributions and the loss of ion gradients is considered to be an important event in the pathophysiology of brain ischaemia and reperfusion injuries (LoPachin et al., 2001). The onset of intraneuronal ion disturbance can be correlated to the development of cellular morphologic alterations and deficits (Taylor et al., 1999; LoPachin et al., 2001), which suggests that loss of ion homeostasis during ischaemia is linked to ischaemia-induced structural and functional changes as shown by degenerative changes in B50 cellular morphology and LDH leakage. The early sign of oxygen-glucose deprivation

associated with brain ischaemia is the reduction in neuronal aerobic metabolism and ATP production. This reduction in energy production leads to loss of active ion transport and subsequent dissipation of transmembrane gradients and membrane depolarization (Vornov, 1998; LoPachin et al., 2001; Kim et al., 2006b). Future studies in this B50 cell model could measure ATP production and normalized to total number of viable cells, hence determine whether ATP production is reduced in hypoxic conditions and the effects of the cannabinoid and opioid agonists.

4.5. Hypoxia-induced cell damage and cell death

The results from this study with B50 cells suggest that hypoxia could have led to B50 neuronal cell death and degeneration in hypoxic condition as shown from the morphological observation and the results from total cell count and viability study, and cellular proliferation (Tables 3.1 and 3.2). The reduction in total cell count, viable cell count and percentage viability could be as a result of oxidative stress and generation of reactive oxygen species during hypoxia and as such resulting in cellular damage of the B50 cells which eventually leads to cellular death and degeneration. The result presented also demonstrated a reduction in the cellular proliferation in hypoxic B50 cells when compared to the normal B50 cells in culture. These reductions may be linked to the exposure of the B50 cells to hypoxia in the culture environment.

These changes are possible because hypoxia has been known to generate reactive oxygen species (ROS) which are by-products of cellular oxidative metabolism (Jayalakshmi et al., 2005). The ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and nitric oxide (NO). However, enhanced production of ROS as seen in hypoxia, overwhelms the antioxidant scavenging capacity of the cells, thus causing oxidative damage to the DNA, lipids and proteins and leads to the development of cellular damage as the case with B50 cells (Lievre et al., 2001). Of all the cells in the body, neurons are highly susceptible to ROS damage and this susceptibility has been implicated in the development of neurodegenerative diseases like Parkinson's disease, Huntington's disease and Alzheimer's disease. There are suggestions that ROS may play an important role in the processes leading to neuronal cell damage due to hypoxia, while Bossenmeyer et al. (1998), had shown

that cultured rat brain neurons on exposure to hypoxia/reoxygenation could induce apoptosis. Lievre et al. (2001), also showed that increased production of free radicals and modification in detoxifying enzymes leads to apoptosis in cultured rat forebrain neurons on exposure to transient hypoxia which can be prevented by antioxidants.

Brain cells have been shown to be specially sensitive to oxidative stress due to the lipid composition of the cell membranes and low level of antioxidant enzymes (Marttila et al., 1988, Jayalakshmi, et al., 2005). Response to oxidative stress in the CNS has been shown to vary according to the various cell types and it was reported that the antioxidant capacity of neurons is lower than that of glia and as such neurons are more susceptible than glial cells to oxidative damage (Maker et al., 1994, Café et al., 1994; Jayalakshmi et al., 2005). Mishra and Delivoria-Papadopoulos (1999), have shown in their study that neurons from rat hippocampus exposed to transient hypoxia showed an increased oxidative stress and decreased antioxidant enzyme system of which the administration of N-acetyl cysteine (NAC), resulted in significant neuroprotection against oxidative stress. NAC has been reported to scavenge free radicals and to augment intracellular levels of glutathione (Jayalakshmi et al., 2005).

4.6. Cellular proliferation and differentiation.

The data presented in Table 3.2 and Figure 3.4 showed that hypoxia affected the B50 neuronal cell proliferation and differentiation when compared with the normal B50 cells while the treatment and pre-treatment of the cells against hypoxia with cannabinoid and opioid agonists improved and increased the rate of proliferation and differentiation of B50 cells in culture (Figures 3.11, 3.13, 3.30 and 3.32). The results also show that cell proliferation was higher in hypoxic B50 cells treated with 100nM Win than those treated with 10nM Win, while those treated with 100nM 2-AG had cell proliferation more that those treated with 10nM 2-AG. These differences in cell proliferation pattern may suggest a concentration dependent effect of the agonists in cell proliferation of B50 cells in culture. The result showed that 100nM Win and 2-AG were more potent and efficacious in increasing cellular proliferation in B50 cells in hypoxia than 10nM Win and 2-AG. In contrast, 100nM Win was more potent than 100nM AEA, while 10nM Win was more efficacious in cellular proliferation in B50 cells treated in hypoxia than 10nM AEA and 2-AG. The results showed that there was a concentration dependent effect in those cells pre-treated against hypoxia with the

agonists. The result showed that 10nM Win was more potent in increasing cellular proliferation in B50 cells pre-treated against hypoxia than 100nM Win, 10nM AEA and 10nM 2-AG, while 100nM Win was more effective in cell proliferation in pre-treatment against hypoxia than 100nM AEA and 100nM 2-AG. These are in support of Lauckner et al. (2005), who had shown a concentration dependent activity of cannabinoid agonists on intracellular calcium concentration in human embryonic kidney (HEK) 293 cells and in cultured hippocampal neurons. There was also a concentration dependent effect on the B50 cells treated and pre-treated with opioid agonists. The results showed that the cells treated with 100μM DAMGO had a higher proliferation than those treated with 10μM DAMGO and 100μM DSLET but had lower proliferation than those treated with 100μM ICI-199441. The result also showed that the cells treated with 10μM DSLET had higher cell proliferation than those treated with 10μM DAMGO and 10μM ICI-199441. The results of pre-treatment of opioid agonists against hypoxia, showed that those pre-treated with 50μM DSLET had higher cellular proliferation than 50μM DAMGO and 50μM ICI-199441 while 50μM DAMGO cell proliferation was higher than those of 10μM DAMGO and 100μM DAMGO in B50 cells in culture. These differences in proliferation could be as a result of differences in the concentration and the mode of action of the different agonists used in the study.

Arimura and Kaibuchi, (2007) have shown that at origin, nerve cells proliferate and differentiate which dramatically changes their shape and establishes two structurally and functionally distinct compartments namely axons and dendrites within a cell. The development of these morphologically and molecularly distinct compartments have recently been shown to be initiated by several signalling pathways (Ciani et al., 2006; Contestabile and Ciani**,** 2004). These pathways are evoked by extracellular signals which have essential roles in a number of aspects of neuronal polarization and functions (Arimura and Kaibuchi, 2007; Krymskaya et al., 2000). Most cells especially those undergoing differentiation, communicate through extracellular proteins. It has been shown that during embryogenesis, a cascade of signalling molecules are initiated between and among cells which instructs cells on their future course of differentiation. Much of this signalling occurs through growth factors, peptide hormones and cytokines, mostly directed by proteins on the cell surface and extracellular matrix (Gen, 2007; Weinstein, 2005). The results presented in this study,

showed that cannabinoid and opioid receptor agonists could have influence over the B50 cell proliferation and differentiation pathway which resulted in the increase in the rate of proliferation and differentiation in B50 cells cultured in hypoxia (Figures 3.13 and 3.32).

Silverman et al. (2001) have shown that hippocampal neurons in culture developed morphological polarity in a sequential pattern in which axons form before dendrites and that molecular differences underlie the functional polarity of these areas especially the relationship between membrane protein polarization and morphological polarization. They showed that in neurons with morphologically distinguishable axons, even on the first day in culture, both axonal and dendritic proteins were polarized. The degree of polarization at these early stages was somewhat less than in mature cells and varied from cell to cell (Villalobo, 2006). This is in support of the results presented in this study which showed that there were differences in cell proliferation and differentiation in B50 cells treated and pre-treated with the different types of agonists which may be due to differences in the concentration and actions of the agonists or differences in the individual cells involved.

The acquisition of a distinct morphological properties by neurons, called polarization, is a critical step in neuronal differentiation. Kinases have been shown to provide entry points for the signalling mechanisms that polarize neurons during the course of their proliferation and differentiation (Kishi et al., 2005; Aguado et al., 2007). A possible explanation why the normal B50 cells have higher rate of cellular proliferation and differentiation when compared with the untreated B50 cells in hypoxia with lower values could be as a result of higher value of phosphorylated kinases present in normal B50 cells as demonstrated from the results of phospho-ERK1/2 assays (Tables 3.19 and 3.20), which promoted more cellular differentiation and proliferation in normal B50 cells than the untreated hypoxic B50 cells in culture. These phospho-ERK1/2 was relatively higher in hypoxic B50 cells treated with cannabinoid and opioid receptor agonists when compared with untreated hypoxic cells except for those treated with 100μM DSLET and 100μM ICI-199441, which could explain why there was a higher rate of cellular proliferation and differentiation in treated cells than in untreated hypoxic B50 cells. The cells treated with opioid agonist showed concentration-dependent effects in that cells treated with lower concentration of the

agonists had a higher level of phospho-ERK1/2 when compared with those treated with higher doses. The cells treated with 10μM DAMGO had higher levels of phospho-ERK1/2 than cells treated with 50μM DAMGO and 100μM DAMGO, while cells treated with 10μM DSLET had higher levels of phosph-ERK1/2 than cells treated with 50μM DSLET and 100μM DSLET. The cells treated with 10μM ICI-199441 had higher levels of phospho-ERK1/2 than cells treated with 50μM and 100μM ICI-199441 respectively. Hence the results revealed a concentration-dependent effects of opioid agonists on the levels of phospho-ERK1/2 in B50 cells cultured in hypoxia. The present findings support the work of Moore and Potter (2001), who have shown a concentration-dependent effects of kappa opioid agonist on intraocular pressure. Hanks and Reid, (2005), had shown that individual opioids may manifest variability in their clinical responses and tolerance and they may induce receptor internalization which are known processes of highly addictive opiate agonists (Kreek, 2000; Compton and Volkow, 2005). The results presented in this study provide a probable explanation for the concentration-dependent action of opioid agonists on phospho-ERK1/2 in B50 cells in culture. The results showed that opioid agonists at low concentrations (10μM) induced a higher level of phospho-ERK1/2 in treated cells in hypoxia and this effect was reduced as the concentration increased (100μM). This may be the situation in which some opiate drugs may be effective at low concentration but at higher concentrations lead to opioid-induced plasticity causing opiate addiction and tolerance (Liao et al., 2007). This is because opioid drugs can be addictive in clinical usage and the dosage to be administered depends on the type of the opioid and situation of the individual involved (Kreek, 2000; Compton and Volkow, 2005). Hence the results presented showed that there were more signalling mechanisms occurring through the proliferation and differentiation pathways in the normal, followed by the treated hypoxic and the untreated hypoxic B50 cells showed the least proliferation and differentiation in culture.

Yoshimura et al. (2006), have shown that neuronal polarity is essential for both structurally and functionally unidirectional signal flow from dendrites to axons. The initial event in establishing a polarized neuron is the specification of a single axon and early in neuronal development, one small neurite becomes differentiated from other neurites to form an axon. The ability of neuronal cells to proliferate, differentiate and polarize is essential for organization of the nervous system and cultured hippocampal

neurons which develop a single long axon and several shorter dendrites that maintain their structural characteristics at the molecular level have been used as a model for neuronal polarization (Yoshimura et al., 2006). During maturation, hippocampal neurons dramatically change their morphology and differentiate into a single axon and many dendrites which form synaptic contacts and establish a neuronal network (Yoshimura et al 2006**;** Dotti et al., 1988). It is these neuronal networks that finally joined together into a specific pattern to form neuronal bundles that ultimately form neural tracts.

The result as presented in Plates 6-9 showed that hypoxia affected neuronal pattern and pattern formation in B50 cells in hypoxia when compared with the normal B50 cells in culture in Plates 3-5. This disorganisation of the neuronal pattern could be as a result of the reduction and disorientation of the proteins necessary for cellular signalling through neuronal cell proliferation and differentiation. Of relevance are the findings of Zhong et al. (2007), who showed that Raf serine/threonine kinases play important role in nervous system development and when these molecules were conditionally eliminated, the result showed markedly reduced phosphorylation of ERK in neural tissues which led to growth retardation. This supports the finding in this present study. Reduced proliferation and differentiation in hypoxic B50 cells could be a result of attenuation in phospho-ERK1/2 in hypoxic B50 cells when compared with normal B50 cells in culture. The importance of this finding is that the reduction in proliferation and differentiation could be corroborated with the reduction in the phospho-ERK1/2 which plays a significant role in the B50 cell signalling mechanisms. Hence, this shows there is a direct relationship between the cellular induced activities like proliferation and differentiation with phospho-ERK1/2 activities, even though Zhang and Fenton (2002), have shown that the proliferation of IL-6-independent multiple myeloma cells does not require the activity of extracellular signal-regulated kinases. This may suggest that the proliferation and growth of IL-6-independent multiple myeloma cells may be independent of ERK activities and as such, other signal transduction pathways may be involved.

4.7 Neuroprotection by cannabinoid receptor agonists

The effect of cannabinoid receptor agonist treatment on the morphology of B50 cells (Plates 9-20), showed a drug-induced changes in the morphology of the B50 cells cultured in hypoxia when compared to the untreated hypoxic B50 cells (Plates 6-8), The hypoxic B50 cells treated with different concentrations of cannabinoid agonists had healthier morphology which is concentration-dependent (Plates 17-19) when compared with untreated hypoxic B50 cells (Plates 6-8). The changes in the morphology could be correlated with changes in proliferation, differentiation and LDH leakage from the B50 cells. The effect of cannabinoid treatment and pre-treatment on proliferation in hypoxia (Tables 3.6 and 3.8), show a concentration-dependent increase in cellular proliferation in cells treated and pre-treated with cannabinoid agonists when compared with untreated hypoxic B50 cells. The effect of cannabinoid agonists treatment and pre-treatment on proliferation in normal B50 cells (Tables 3.5 and 3.7), show a concentration-dependent increases in cellular proliferation. The results also revealed differences in cell proliferation from the different cannabinoid agonists used. The cells treated with Win and AEA, showed a concentration-dependent increase in cell proliferation in which cells treated with 100nM of the agonists had higher cellular proliferation compared to those of lower concentration of the agonist (Table 3.6) in hypoxia, while the cells treated with 2-AG had a concentration-dependent decrease in cellular proliferation whereby the low concentration of the agonist produced higher cellular proliferation in B50 cells cultured in normal (Table 3.5). The result also showed that the proliferative effects of the cannabinoid agonists was not completely abolished by the cannabinoid antagonists (AM251) since some of the effects still show significant increases in cell proliferation in the presence of the antagonist, suggesting that the actions of the agonists may be mediated through both receptor- and non-receptor-mediated pathways in the same cell. The mediation through the cannabinoid (CB_1) receptors in this process occurs via the activation of $G\alpha_{i/o}$ proteins. Aguado et al. (2007) have shown that the CB_1 receptors mediate excitotoxicity-induced neural progenitor proliferation and neurogenesis. It has been shown that the endocannabinoid system exerts an important neuromodulatory function in different brain tissues and is known to be involved in the regulation of neural cell fate (Galve-Roperh et al., 2007). There is the presence of a functional endocannabinoid system in neural progenitor cells that participates in the

regulation of cell proliferation and differentiation which play a regulatory role in neurogenesis (Galve-Roperh, et al., 2007).

The endocannabinoid system has been shown to affect early progenitors and this extends to the regulation of neuronal migration and the attainment of the morphological, physiological and molecular characteristics that occur during terminal neuronal differentiation (Harkany et al., 2007). It has also been shown that AEA and Win, with brain-derived neurotrophic factor, a pro-differentiating neurotrophin, induce the migration of GABA-containing interneurons that undergo migration to populate the embryonic cortex (Harkany et al., 2007). The activation of the CB_1 receptors in which downstream signalling events such as proliferation and differentiation occur, exert profound effects on neurite outgrowth and synaptogenesis (Harkany et al., 2007). AEA and Win have been shown to strongly inhibit neurite formation and elongation in GABA-containing interneurons (Berghuis et al., 2004; Berghuis et al., 2005). In N1E-115 neuroblastoma cells, AEA and HU210 reduce the rates of neurogenic differentiation by inducing cell rounding, and neurite remodelling and retraction through the recruitment of the Rho family of small guanosine triphosphatases (Jaffe and Hall, 2005). The cannabinoid agonist, HU210 promotes neurite outgrowth in Neuro 2A cells by the $G\alpha_{o/i}$-mediated degradation of Rap–GAPII and the subsequent activation of Rap1 (Jordan et al., 2005).

The results of the present study showed some consistency with other studies showing that the different cannabinoid receptor agonists used acted through the G protein coupled receptors (GPCRs) to mediate cellular activities, functions and alterations via the intracellular second messenger pathway. These cellular activities such as proliferation and differentiation were shown to increase in the cells treated with the cannabinoid agonists when compared with the untreated hypoxic B50 cells in culture. The most striking and important aspect of the cannabinoid-induced action was the fact that they could be diluted to lower concentrations in the medium during treatment and they still provided protection and increased cellular activities over extended periods of time (Zhuang et al., 2001).

The effect of cannabinoid treatment on LDH release from B50 cells (Table 3.10), showed a change when compared to the untreated hypoxic cells and the change in

LDH release was concentration-dependent. The results also showed differences in LDH release between the cannabinoid agonists (Win and AEA) used, in which there was increase in LDH release from B50 cells with increase in the concentration of Win while there was a decrease in the LDH leakage with increase in the concentration of AEA. Hence there was an inverse relationship between the LDH leakage in B50 cells in hypoxia in which the LDH release was increasing with increase in Win concentration while decreasing with increase in AEA concentration. This difference in LDH release could be as a result of the difference in the concentrations of the agonists used, because these agonist though mediating their effects through CB_1 receptors, may have other systems involved in the overall protective effects of the cannabinoid agonist system. Kim et al. (2005) have shown there was a reduction in LDH release from primary cortical cell culture in the presence of increasing concentrations of the Win with 50% inhibition of the effect of $FeCl_2$ at 100nM Win, while AEA also decreased $FeCl_2$ toxicity by producing half-maximal inhibition at 300nM AEA. The involvement of CB_1 receptors in cannabinoid neuroprotective effects of Win and AEA, was because of the ability of the cannabinoid antagonists to inhibit the protection from the agonists, thus pointing to a $G_{i/o}$-mediated mechanism.

The reduction in LDH release could be due to the protection afforded the cells by the cannabinoid from the oxidative stress caused by the hypoxia. This is because exogenous cannabinoids have been shown to protect against neurotoxicity in a number of different cellular, animal and human experimental models (Pryce et al., 2003; Davies et al., 2002; Wang et al., 2007b). Zhang et al. (2005) have demonstrated that cultured rat hippocampal neurons were protected from excitotoxic insults by pre-treatment with either Δ^9-THC or Win and these compounds were effective in preventing cell death even if administered prior to the neurotoxic exposure. Cannabinoid protection has been demonstrated *in vivo* with respect to neurodegenerative changes resulting from experimental ischaemia (Sánchez et al., 1998; Leker et al., 2003). In the present study, cannabinoid agonist treatment with Win, AEA and 2-AG was shown to be neuroprotective by the reduction of LDH leakage from the B50 cells. This is because LDH leakage has been shown to be a reliable method of accessing neuronal injury and damage in culture model (Zhang et al., 2006), and when the LDH leakage from the cells is reduced, cells were improved morphologically and functionally.

However, the results presented show some disparities in the concentration-dependent agonist response to hypoxia in that it was expected that the drugs would have more beneficial effects at higher concentrations which was not seen to be so as shown in the results (Table 3.10), with 100nM Win treatment having 527% of LDH release which is even more that of the hypoxic untreated cells with 440% when compared with the normal cells with 100% LDH leakage. The effect of cannabinoid receptor agonist pre-treatment on LDH release (Table 3.13), of which the B50 cells pre-treated with had 10nM Win (99%), 50nM Win (404%) and 100nM Win (447%) had LDH release.

Three mechanisms could be attributable to the reactions of the these drugs used in the study. The G protein coupled receptors involved could be over activated, desensitized or saturated. Thus at higher concentrations of cannabinoid administration, the receptors could either become over-activated, desensitized or become saturated and hence leading to the higher concentrations of the drugs not resulting in higher or elevated response to the drug action. It could also be that at higher concentrations of the drugs, the receptors become highly activated and hence cell death could result from that leading to elevated LDH release from the B50 cells in culture. The mechanism involved could also be that the receptor stimulation by higher concentrations of the agonists evoke Ca^{2+} release from the endoplasmic reticular stores followed by Ca^{2+} influx through store-operated Ca^{2+} channels in the plasma membrane. Chang et al. (2007) had shown that at a single cell level, Ca^{2+} influx was related to agonist concentration in an all-or-none manner in mast cells and that increasing the agonist concentration, recruited more mast cells but each cell responded by generating all-or-none Ca^{2+} influx. This effect could also be an example of a specialized functional homologous or heterologous desensitization of the CB_1 receptors in higher concentrations of the agonists which induces interference on the receptor-induced activities. This is because Itzhaki Van-Ham and Oron (2005), have shown that $G\alpha_o$ G-proteins mediate both homologous and heterologous rapid desensitization of responses mediated by G-protein-coupled receptors coupled to the phosphoinositide phospholipase C-inositol trisphosphate-Ca^{2+} pathway in Xenopus oocytes. Also the higher concentrations of these drugs could actually be toxic and hence result in toxic death of the cells involved leading to increased LDH leakage from the B50 cells in such media (Downer et al., 2003; Sarne and Mechoulam, 2005).

Zhang et al. (2001) have demonstrated that cannabinoid receptor mediated neuroprotection is sensitive to intracellular calcium levels, hence cannabinoids may act by reducing or blocking the release of intracellular calcium $(Ca^{2+})i$ under neurotoxic conditions such as hypoxia. This intracellular calcium mechanism is possible in the case of B50 cells in hypoxia treated and pre-treated with different concentrations of cannabinoid agonists. This is because during hypoxia in B50 cells, there may be an increase in $(Ca^{2+})i$ influx into the cells through the ionotropic channels in the cell membrane leading to increased Ca^{2+} accumulation that consequently results in the death of the cells. This cellular damage leads to increased LDH leakage from the cells into the media. Hence cannabinoid agonists could act by blocking the ionotropic channels which blocks the influx of the calcium ions into the intracellular surfaces thereby preventing the cells from calcium ion intoxication and consequent death of the cells. When this protection is proffered, it leads to the reduction of cell death in hypoxia which is correlated by the reduction of LDH leakage from the B50 cells into the culture media.

One of the factors underlying the cannabinoid induced changes is the increase in cAMP levels in the cannabinoid treated hypoxic groups when compared to the hypoxic untreated B50 cell groups. This may be due to the cannabinoid agonist-induced stimulation of CB_1 receptors leading to cannabinoid receptor-mediated stimulatory effect on cAMP accumulation in the cells through $G\alpha_s$ (Glass and Felder**,** 1997). The cannabinoid-induced increase in cAMP accumulation in B50 cells when compared to untreated hypoxic cells, may lead to the increased down-stream signalling activities such as proliferation and differentiation in cannabinoid-treated cells, through a combination of molecular signalling pathways in B50 cells (Yao et al., 2006). Guo and Ikeda (2004) have shown that cannabinoid mediate alterations in intracellular calcium $(Ca^{2+})i$ levels via an intracellular second messenger pathway which provides a basis for cannabinoid effects on intracellular calcium ion mediating the neuroprotection. The significant feature of this finding is that protection for the B50 cells can be triggered by relatively low concentrations of cannabinoids acting on the CB_1 receptors like 10nM Win. Neuroprotection from reduced $(Ca^{2+})i$ can persist for relatively longer periods due to the extended recovery time of protein kinase A to constitutive levels (Zhuang et al., 2005).

It is shown in the results (Table 3.13), that since the cannabinoid pre-treatment effects were concentration-dependent with respect to the magnitude of the excitotoxic insult (LDH release), there exists considerable potential for adjustment of these concentrations with respect to the possible potential therapeutic application of these potent synthetic cannabinoid compounds in hypoxia-related conditions (Zhuang et al., 2005). Cannabinoid have been shown to protect neurons from toxic insults such as glutamatergic excitotoxicity, ischaemic stroke, hypoxia, trauma, oxidative stress and ouabain-induced secondary excitotoxicity (Zhang, et al., 2005; Smit and Iyengar, 1998). Most of these protectant effects appear to be mediated by the activation of the cannabinoid (CB_1) receptors (Grundy, 2002; Machoulam, et al., 2002; Biegon, 2004).

The effect of cannabinoid agonists was reversed by the action of the antagonists (Table 3.11), using LDH release from the cortical B50 cells in culture. The result showed that the cannabinoid agonists (10nM Win, 50nM Win, 50nM AEA and 100nM AEA), were able to reduce the levels of LDH release from the B50 cells in hypoxia. These effects were blocked by the cannabinoid receptor antagonists AM251 and AM630, suggesting both cannabinoid receptor- and non-receptor-mediated mechanism. The cannabinoid agonist Win was shown to reduce neuronal cell death in murine cortical cultures treated with 20μM NMDA and its protective effect was attenuated by the CB_1 receptor antagonist SR141716A (Kim et al., 2005), even though the results presented (Table 3.11) for the action of antagonists against the agonists may show some disparities in the LDH leakage in the treatment with the agonists and antagonists during hypoxia in B50 cells. One of the most promising potential medical applications of cannabinoids involves their ability to protect cells from a variety of toxic events (Guzman et al., 2001). Cannabinoids have been reported to protect neurons from death caused by glutamatergic over-stimulation, ischaemia and oxidative damage (Hansen et al., 2002). These effects could have been due to the cannabinoids having multiple effects on the G proteins mediated responses (Slessareva et al., 2003). The differences between the effects observed in different models may be related to the cell type or model system used and the differences in the toxic events which have been employed (Chen, et al., 2005; Drysdale and Platt, 2003; Zhang et al., 2005).

The results show that some antagonists completely reversed the action of the agonists while others did not. This effect can be seen with the cells treated with 10nM AM251/10nMWin, in which the LDH released was lower than that released by the untreated hypoxic B50, while the cells pre-treated with 10nM AM251/10nM Win showed more LDH release. These disparities may be due to the antagonists not being in the right amount and concentration to counter the effect of the agonists or the reduction in LDH release by Win may not be entirely mediated through the CB_1 receptors. It was because of these differences that concentration-response experiment was conducted using a standard concentration of the cannabinoid agonist (10nM Win), with varied concentrations of the antagonist (AM251), in both treatment and pre-treatment groups (Tables 3.16 and 3.17). The result showed that the treatment of B50 cells in hypoxia with 10nM Win required 5nM concentrations of the antagonist (AM251) to bring about the complete inhibition of the agonist (10nM Win) activity in reducing the LDH release from B50 cells while pre-treatment with 10nM Win used 1nM concentrations of the antagonist (AM251) to bring about complete blockage of the agonist (10nM Win) action of LDH inhibition of B50 cells cultured in hypoxia.

This concentration-dependent response of the antagonist to the agonistic action may suggest that antagonists does not function on equivalent concentrations, and as such, could not function effectively on a one to one basis. It was for this reason that differences in the antagonist reaction to the agonist action regarding LDH release from B50 cells were evident. The result showed that different concentrations of the agonist require different and specific concentration of the antagonist to bring about effective antagonism. Also if the concentrations are at the right proportion, the condition of the system in which the agonists and the antagonists interact is important for effective activity of both the agonists and the antagonists. The results showed that less concentration of antagonist was required to reverse the action of the agonist during pre-treatment with the drugs when compared with the treatment with the agonist. This means that the smaller amount of a drug can produce an effective response when introduced as a preventive measure rather than during treatment. Hence drugs used for inoculation in cases of vaccinations are usually administered at very small quantity which would produce a maximal effect, but when the disease is present, larger concentration of the drug will be required to produce the desired treatment from the drug.

4.8 Neuroprotective effects of opioid receptor agonists

Five independent approaches were used to determine neuronal injury and damage in this study namely, morphological-based same field assessment, LDH assay, live/dead staining using trypan blue exclusion method, neuronal differentiation using DbcAMP and neuronal proliferation assay method. The results of the study show groups of dead and degenerating B50 cells in hypoxia (Plates 23, 25 and 26), when compared with the normal cells (Plates 22 and 24). When the hypoxic B50 cells were treated with different concentrations of opioid (Mu, delta and kappa) agonists and compared to the hypoxic untreated B50 cells, the results showed healthier looking cells with fewer degenerating cells. The opioid receptor agonists (DAMGO, DSLET and ICI-199441), may have provided protection to the B50 cells against the hypoxic injury and damage (Plates 27-29). The results of the effect of hypoxia on total cell count and viability, cellular proliferation and the LDH release are shown in Tables 3.1 and 3.2. These studies draw a detailed time course effect of hypoxia on total cell count and viability, cellular proliferation and LDH release in cultured B50 neuronal cells based on neuronal age and hypoxic duration, which demonstrated that cultured B50 neuronal cells are more vulnerable to hypoxic stress with maturation.

The result of opioid agonists treatment and pre-treatment on the proliferation of B50 cells in hypoxia (Tables A14 and A16), showed an increase in neuronal proliferation when compared with untreated hypoxic cells. This increase could be as a result of the opioid agonists mediating cellular activities like proliferation and differentiation through increases in cAMP signalling activities leading to increased cellular signalling through proliferation and differentiation activities. Tencheva et al. (2005), had observed that opioid agonists acting via specific opioid receptors have been shown to influence neural cell proliferation, and Law et al. (1997), had shown that chinese hamster ovary (CHO) cells transfected with δ-opioid receptor cDNA resulted in an agonist concentration-dependent potentiation of foetal calf serum-stimulated cell proliferation. The effect of μ, δ and κ opioid agonists on proliferation was abolished by the selective mu, delta and kappa antagonists (Table A17), suggesting that these effects were mediated through the μ, δ and κ opioid receptors in B50 cells in culture. Since this potentiation of cellular proliferation by the opioid agonists (μ, δ and κ) was antagonized by the selective antagonists, thus indicating that $G_{i/o}$ was involved in this action. However this opioid agonist potentiation of cellular growth and proliferation

apparently was dependent on the level of agonist concentrations used in that, Law et al. (1997), had suggested that δ- and μ-opioid receptors in CHO cells activate similar but divergent second messenger pathways, resulting in differential regulation of cell growth, proliferation and differentiation. This is in agreement with the findings from the present study which show that the increase in B50 cell proliferation induced by the opioid agonist was concentration-dependent. The results show that cells treated with the agonist DAMGO and ICI-199441, had increases in cell proliferation as the concentration of the agonists increases, while those treated with DSLET demonstrated cell proliferation decreases with increase in concentrations of the agonist. The effects of the opioid agonist pre-treatment against hypoxia in B50 cells show that the cell proliferation was decreased with increases in the concentration of DAMGO and DSLET while there was no change in cell proliferation in those pre-treated with ICI-199441. The results show some differences in the overall activity of the agonists and the antagonists suggesting that multiple processes may be involved in the regulation of opioid receptors (Tencheva et al., 2005).

The effects of opioid agonists (DAMGO, DSLET and ICI-199441) administration on LDH release from B50 cells in hypoxia (Tables A1 and A3), showed significant reductions in LDH leakage in hypoxia-treated B50 cells in culture while the pre-treated B50 cells with opioid agonists showed more LDH leakage when compared with the treatment group. The use of LDH assay has a unique advantage of using the same culture dish which provides relatively accurate time course data for hypoxic duration and opioid agonist treatment experiments. The release of intracellular LDH into the extracellular fluids has been documented as a reliable indicator of neuronal injury and damage (Zhang et al., 2006). The results showed that there was a concentration-dependent decrease in LDH release from treated B50 cells in hypoxia. The cells treated with DAMGO and DSLET had LDH release decreased with increase in the concentration of the agonists while ICI-199441, had LDH release increased as the concentration of the agonist increases. The effect of the agonists in lowering the LDH release from B50 cells was blocked by the selective mu, delta and kappa (CTAP, ICI-174864 and NOR) opioid antagonists suggesting that the effect of inhibiting the LDH release from B50 cells was mediated through the (μ, δ and κ) opioid receptors, indicating the involvement of $G_{i/o}$ in the protective activities of the opioid receptor agonists. There was some relative differences in the actions of the

agonists and antagonists like in the pre-treatment with 100μM ICI-199441, in which the LDH release was lower than that of 10μM ICI-199441 (Table A3), treatment with 100μM DSLET was higher than that of 50μM DSLET (Table A1), and antagonist pre-treatment with 10μM DAMGO and 10μM DSLET had LDH release lower than those of the agonist involved (Table A4). Thus indicating that there may be other systems and processes involved in the regulation of opioid receptor activities in B50 cells in culture. These multiple processes could be as a result of interaction between the various opioid receptor subtypes involved, since it has been shown that the opioid receptor subtypes share a close homology in their sequences (Mayer et al., 2003). There may be the involvement of endogenous opioids in the system, which have been shown to be released during hypoxia (Zhang et al., 2000). Also there may be the involvement of interaction between the opioid agonists, the endogenous opioid and the endogenous cannabinoid system present in the cells (Schoffelmeer et al., 2007). This is because Zani et al. (2007) have demonstrated that opioid agonist (AM 404) and cannabinoid agonist (Δ^9-THC), reduced neuronal damage caused by bilateral carotid occlusion in gerbils and that this protection was mediated through an interaction between CB_1 and opioid receptors.

Hong et al. (2004) have shown that although δ-opioid receptor inactivation reduces LDH activity in normoxic neurons, neither δ-opioid receptor activation nor inactivation induces any appreciable effect on LDH activity in the cortical neurons under hypoxia and as such, other signal transduction pathways such as the glycolytic pathway, may play an important role in neuroprotection. Zhang et al. (2002) had shown clearly that the modulation of δ-opioid receptor, but not μ- and κ-opioid receptors, plays a major role in neuroprotection in both normoxic and hypoxic environments. Though the mechanism involved in this neuroprotection is not well understood, Zhang et al. (2002), speculated that this phenomenon may be linked to the role of δ-opioid receptors in selective regulation of G proteins, excitatory neurotransmitter release, glutamate receptor stimulation, and Ca^{2+} homeostasis. The activation of opioid receptor subtypes may have a wide range of clinical applications in treating and preventing acute and chronic hypoxia-related impairments. Further research in this area is necessary to develop a better understanding of opioid receptor activities and the pathways involved in their neuroprotection.

The use of LDH release as an index of neuronal injury does not provide direct assessment of the percentage of neurons injured and/or dead in culture. Hence the use of additional experimental approaches which include morphological assessments, live/dead trypan staining for total cell count and viability and proliferation assay was used to assess neuronal injury more directly. It has been shown that during hypoxia, glutamate is expelled from neurons leading to over-stimulation of glutamate receptors and subsequent injury and death of the neurons (Figure 4.1) (Haddad and Jiang, 1993; Nyakas et al., 1996). Since Zhang et al. (2002), had shown that because glutamate receptor expression increases during development, and sensitivity to glutamate excitotoxicity increases with neuronal maturation, the observed differences in hypoxic susceptibility between the neuronal ages in this study may be associated with the developmental increase in glutamate toxicity (Choi and Rothman, 1990).

This study has demonstrated that stimulation of opioid receptors, proffers some protection by reducing neuronal cell injuries and deaths after treatments in hypoxic conditions but the benefit is reduced substantially with prolonged exposure durations and higher concentrations of the drugs. This supports the finding that the longer the neurons stayed in the culture media both in the normal and in the experimental hypoxic groups, the greater the extent of the neuronal cell injuries and death. A possible explanation for this phenomenon is that prolonged hypoxia may cause a significant release and accumulation of endogenous glutamate which causes glutamate-induced toxicity and hence death of the cells (Nyakas et al., 1996). Another alternative explanation is that prolonged hypoxia causes the release and accumulation of endogenous opioids which saturates opioid receptors in these neurons. Hence the positive effect of the opioid agonists decreases with the increase in the time the cells stay in the medium which may result in desensitization of the receptors and the reduced effect of the action of the drugs (Mao et al., 2002; Wallace et al., 2006).

It has been shown that in response to short-term hypoxia, the level of enkephalins, the endogenous agonists for opioid receptors sharply increases (Zhang et al., 2002; Khasabova et al., 2002). This showed that cortical neurons may release opioids during normal function and in response to hypoxic stress as a mechanism of self-protection against injury (Ma et al., 2005; Wallace, et al., 2006). Zhang et al. (2002), showed that because of high levels of endogenous opioids which may already be present in the

culture media after prolonged exposure to hypoxia, adding more agonist may not increase the protection. On the other hand, desensitization of the receptors may have occurred due to prolonged treatment of the cells with the agonists in conjunction with the endogenous opioid release during chronic hypoxia. Zhang et al. (2006), have shown that cortical neurons are highly susceptible to opioid receptor inhibition, which causes serious neuronal injury especially during hypoxic stress.

However, the greater injury was observed in 144 hours cultured B50 neurons than in 48 hours cultured B50 neurons in both normoxic and hypoxic conditions which suggests that maturational differences exist between these age group of neurons. This supports the work of Zhang et al. (2000), which showed that opioid receptor expression increases significantly with development in both brain and cultured neurons. This is because the opioid receptor density increases with increase in the age of the neurons. The more mature neurons may have greater dependence on this pathway to maintain neuronal function and therefore may be more susceptible to neuronal injury with opioid receptor inhibition. This observation suggests that the increase in opioid receptor agonist may compete with opioid receptor antagonist in terms of opioid receptor binding and thus reduce neuronal injury induced by opioid receptor inhibition during prolonged hypoxia (Wallace et al., 2006). This is in support of the findings in this study in which at higher concentration of the agonists and antagonist DAMGO/CTAP (100μM), DSLET/ICI-174864 (50μM) and ICI-199441/ NOR (50μM) during the pre-treatment against hypoxia, which led to complete inhibition of the agonists activities, resulting in increased LDH release in cultured B50 cells in hypoxia.

Another issue is whether the differences in expression levels of the various opioid receptors account for the observed phenomenon in cortical neurons. Past studies have demonstrated that μ-opioid receptors are present at similar or even higher densities than δ-opioid receptors in mammalian cortex, although κ-opioid receptor density is slightly lower (Zhang et al., 2006). This implies that the relative distribution and expression levels of opioid receptor subtypes within the cortex as a whole may not be a key factor in the observations shown in this work. This is because the results presented in this study show that there was no observed differences in the receptor

expression densities of μ-opioid receptors in normoxic, hypoxic and hypoxic treated B50 cells in culture.

The mechanisms of opioid receptor neuroprotection activity may involve the regulation of specific G proteins, ion channels mainly Ca^{2+} and K^{+} channels and excitatory neurotransmitter release. It has been shown that intracellular Ca^{2+} levels are elevated during hypoxic exposure leading to irreversible cell injury while the inhibition of Ca^{2+} currents by opioid receptor stimulation by DAMGO, DSLET and ICI-199441, may serve as a neuroprotective mechanism in preventing Ca^{2+} overload (Andersen, 2004; Bossy-Wetzel et al., 2004). Also opioid receptor regulation of glutamate signalling may be involved in normal function and protection of neurons (Ma et al., 2002, Mao et al., 2005). It has also been shown that opioid receptor agonists have the ability to reduce neuronal over-stimulation by blocking glutamate excitation (Zhang et al., 2002), and this could be the situation with the protection proffered by the μ-, δ- and κ-opioid agonists used in this study. This mechanism of cellular regulation may be utilised during normal cell functioning and in response to environmental stress like hypoxia (Andersen, 2004). Zhang et al. (2006), have shown that the inhibition of opioid receptors in normal neurons may lead to substantial injury in the neurons by the loss of inhibitory regulation of the excitatory neurotransmitter release and/or receptor excitation.

The δ-, μ- and κ-opioid receptors have many similarities such as seven transmembrane domains existing as 60% identical sequences and being coupled to $G_{i/o}$ proteins. They are also similar in several of their regulatory targets including adenylyl cyclases, protein kinases and certain Ca^{2+} and K^{+} channels (Bickler et al., 2004; Zhang et al., 2006). One possible explanation for the differences in neuroprotective abilities and actions of these three different opioid receptors is that the individual opioid receptors regulate different effectors thereby eliciting different responses (Tables A1 and A2). Connor and Christie (1999), had proposed that because of the common features of opioid receptors, the selectivity of these receptors for eliciting specific pathways does not lie in the differences between each opioid receptor subtype but in their association with other divergent types of G proteins. It was observed that each opioid receptor subtype also preferentially couples to specific G proteins apart from the $G_{i/o}$ proteins that they generally couple (Zhang et al., 2002).

Examples of this preferential coupling include as seen in δ-opioid receptors which are more efficiently coupled to $G\alpha_{16}$ protein than either μ- or κ-opioid receptors (Lee et al., 1998). Also μ-receptor agonist (DAMGO) has been reported to have a selective coupling to $G\alpha_{i1}$ and $G\alpha_{oA}$ opioid receptors than other opioid agonists (Saidak et al., 2006). This shows that the opioid receptor agonists have selective activation of G-proteins in response to opioid receptor activation. This preferential coupling to other G protein subtypes may be the reason for the observed differences in the effect of the opioid agonists on the B50 cells treated in hypoxia. The activation of μ-, δ- and κ-opioid receptors by the agonists may have a wide range of clinical implications in the treatment of hypoxia-related impairments and may form the basis for the development of new neuroprotective and therapeutic drugs useful for the treatment of stroke and other neurodegenerative pathologies.

4.9. Intracellular cAMP

The results presented (Table 3.18), showed that there was a decreased cAMP release in hypoxic B50 cells when compared with normal B50 cells, which is in agreement with the findings of Cooper and Crossthwaite (2006), which showed that as the concentration of intracellular cAMP increases, it leads to activation of cAMP-dependent protein kinases and phosphorylation of targets which could lead to coordinated activities of the B50 cells such as cellular growth, proliferation and differentiation. The intracellular concentration of cAMP could be increased or decreased by a variety of ligands and hormones and such fluctuations affect a variety of cellular processes and activities. One prominent and important effect of elevated concentrations of cAMP is activation of cAMP-dependent protein kinases (Cooper, 2005). The results presented in this study showed that treatment of B50 cells in hypoxia with cannabinoid receptor agonists increased cAMP release in B50 cells in culture when compared with untreated hypoxic B50 cells with the exception of the cells treated with 10nM AEA and 50nM AEA in which the cAMP release was the same or less than that released by the untreated hypoxic cells. The changes in cAMP release during cannabinoid agonist treatment show in the case of cells treated with the cannabinoid agonist (2-AG), that the level of cAMP release was decreased with increase in the concentration of the agonist, hence those treated with 10nM 2-AG had higher cAMP release than 50nM 2-AG while 100nM 2-AG had the least cAMP release from B50 cells treated in hypoxia. The results also showed that the cells treated with Win and

AEA agonists had cAMP release increased with increase in the concentration of the agonists hence, the cells treated with 100nM Win and AEA had higher cAMP release when compared with the low concentration of the agonists. The results (Table 3.19) of the opioid treatment on cAMP release, show increase in cAMP release when compared to untreated hypoxic B50 cells in culture. The results show in the case of ICI-199441 that the cAMP release was decreased with increase in the concentration of the agonist while in the case of DAMGO and DSLET, the cAMP release was also decreased with increase in the concentration of the agonists with some differences among the agonists. The results showed that the cannabinoid and opioid receptor agonists' administration to B50 cells in hypoxia resulted in the increase in cAMP release when compared with untreated hypoxic cells in culture (Tables 3.18 and 3.19).

The regulation of intracellular concentrations of cyclic AMP has been shown to be largely due to the controlling effect of adenylyl cyclase (Cooper et al., 1995; Houslay and Milligan, 1997). It has been shown that when adenylyl cyclase (AC) is activated, it catalyses the conversion of ATP to cAMP which leads to an increase in intracellular levels of cAMP (Cooper and Crossthwaite**,** 2006). Hence there is a special relationship between the intracellular levels of cAMP and AC, the higher the level of AC that is activated, the higher the level of intracellular cAMP released into the cytoplasm for signalling activities of the cells assuming availability of ATP is constant. Thus the results presented in this study, showed that the higher the amount of cAMP released by the B50 cells in culture, the higher the signalling activities exhibited by the cells. These signalling activities such as proliferation and differentiation were more in the normal cells followed by the hypoxic treated cells while the untreated hypoxic B50 cells had the least signalling activities, which correlated with the cAMP release from the cells in this study. The AC enzymes differ considerably in regulatory properties and have a very complex model of interactions that regulate cAMP production which affect the physiological state of the cells (Cooper, 2005; Cooper and Crossthwaite 2006).

Cannabinoids have been shown to exert many of their effects through activation of G_i-protein-coupled receptors and functional coupling of CB_1 receptors have been demonstrated in the inhibition of adenylate cyclase and voltage-dependent calcium channels via pertussis toxin-sensitive G-proteins (Felder et al., 1992). Both dopamine (D_1

and D_2) receptors have also been shown to couple adenylate cyclase via G-proteins. Dopamine (D_1) receptors stimulate adenylate cyclase via G_s-proteins, whereas dopamine (D_2) receptors inhibit adenylate cyclase via G_i-proteins and there have been associations between the actions of cannabinoids and dopamine (Glass et al., 1997). It has been reported that concurrent activation of CB_1 and D_2 receptors results in an increase in cAMP accumulation in contrast to the inhibition of cAMP accumulation normally observed with activation of either of the receptors alone (Glass and Felder, 1997). The CB_1 receptor couples to a G_s-protein indicating that cannabinoid receptor function may be more complex than the simple G_i linkage that is generally known (Howlett, 2004). The CB_1 receptor interaction with G_s has been demonstrated in Chinese hamster ovary cells stably expressing recombinant human CB_1 receptors (Bonhaus et al., 1998).

The increase in the cAMP release in response to the treatment with cannabinoid agonists presented in this study may be caused by the coupling of the CB_1 receptors to both G_i which mediates the inhibition of adenylate cyclase, and to a stimulatory component G_s, resulting in increases in cAMP accumulation in cannabinoid-treated B50 cells in hypoxia. In the study with primary striatal cells, Glass and Felder (1997), showed that the inhibitory effect on cAMP accumulation appear to dominate over the stimulatory component because the stimulatory component can only be observed when the inhibitory component was reduced. However, some of the treated cells (50nM AEA), showed reduced cAMP levels when compared with hypoxic untreated cells. This reduction in cAMP accumulation could be due to the direct effect of the drug thereby promoting the inhibition of adenylate cyclase release from the B50 cells through the G_i causing a reduction in cAMP release. Another explanation could be that since the cortical neurons are found in association with the striatal dopamine neurons that concurrent activation of CB_1 and D_2 receptors could result in an increase in cAMP accumulation as shown with the B50 cortical neurons treated with cannabinoid agonists in hypoxia. However, the increase in the accumulation of cAMP occurred only at higher concentrations of the cannabinoids (Win and AEA), and was likely attributable to the membrane-perturbing effects of these hydrophobic compounds (Felder et al., 1992). It has been shown that treatment of striatal neurons with pertussis toxins prevented the inhibition of cAMP accumulation by D_2 receptors but unmasked a cannabinoid receptor-mediated stimulatory effect on cAMP accumulation (Felder et al., 2006). Similar augmentation of cAMP

accumulation was also observed by Glass and Felder (1997), in Chinese hamster ovary cells transfected with, and stably expressing, the CB_1 receptors and this stimulation of cAMP was not Ca^{2+}-sensitive, since the treatment of the pertussis toxin-treated cells with cholera toxin before CB_1 receptor activation amplified the stimulatory pathway, suggesting that this response was mediated through a G_s-type G-protein.

The results presented in this study which showed an increase in cAMP release as a result of cannabinoid agonist treatment, resulting in the activation of the CB_1 receptors leading to the stimulation of cAMP accumulation, may be mediated through the stimulatory $G\alpha_s$ proteins. This is because $G\alpha_s$ and forskolin have been shown to be synergistic in their activation of adenylate cyclase (Barovsky and Brooker, 1985). Also the increased cAMP accumulation was observed when cannabinoid agonist, HU210, and forskolin were added together, compared with the level of stimulation observed when each agent was added alone (Glass and Felder, 1997). The mechanism involved in this could be that the $G\alpha_s$ subunits only become available as a substrate as they are released from the heterotrimeric G-proteins after receptor stimulation, resulting in the amplification of agonist-dependent increases in cAMP accumulation. This shows that at higher concentrations of the cannabinoid agonists (Win and AEA), there was a reversal of the inhibition of cAMP accumulation observed at lower concentrations, suggesting an underlying stimulatory component. It has been shown that dopamine (D_2) agonists through $G_{i/o}$ oppose the action of low concentrations of CB_1 agonists acting through $G\alpha_s$ to modulate cone cell membrane currents, suggesting a role in shaping the cone light response and/or sensitivity to changes in ambient light conditions in cone cells of goldfish (Fan and Yazulla, 2004). It has been shown that cannabinoid receptors couple to both G_s and G_i proteins and can consequently stimulate or inhibit the formation of cAMP. Bonhaus et al. (1998), had shown that the cannabinoid agonists (anandamide and CP-55,940) were markedly less efficacious in stimulating the accumulation of cAMP than in inhibiting its formation. This receptor mediated stimulation of adenylyl cyclase revealed differences among agonists which support the finding from the present study that AEA has the least stimulatory effect on cAMP accumulation in B50 cells treated in hypoxia. Hence taken together, the findings from this study demonstrate differences among cannabinoid receptor agonists in their activation of intracellular cAMP release and as such the transduction pathways of cAMP in B50 neuronal cells may differ with these agonists. Another mechanism

resulting in the cannabinoid receptors couple to both G_s and G_i proteins and can stimulate or inhibit the formation of cAMP, is the differences in the relative and intrinsic activities of CB_1 receptor agonists. The cannabinoid receptor agonists may have different affinities for G_s- and G_i-coupled CB_1 receptors in B50 cells in which a higher affinity towards G_s proteins will result in stimulatory cAMP effects but an affinity towards G_i proteins will lead to inhibitory cAMP effects as in the results of this study. This is because CB_1 receptors have been shown to predominantly couple G_i receptors but cannabinoid-induced tolerance is associated with a G protein coupling switch from the inhibitory G_i protein to the excitatory G_s protein (Paquette et al., 2007).

The results presented showed increase in cAMP release following treatment with μ, δ and κ opioid receptor agonists in B50 cells cultured in hypoxia. This is in agreement with the findings of Russell and Potter (2001), which showed the stimulation of cAMP levels in the iris-ciliary body by bremazocine suggest that a κ-agonist has dual effects in inhibiting isoproterenol-stimulated cAMP production at high concentrations and in the absence of isoproterenol, enhancing cAMP levels at low concentrations of bremazocine. Dziedzicka-Wasylewska and Przewlocki (1995), have shown that the κ-opioid receptor agonist (U50,488H) and μ-opioid receptor agonist (morphine), affected opioid receptor activation *in vitro* by increasing the cAMP level on the slices of hippocampus obtained from kainic acid-treated rats. The increase in cAMP level in B50 cells treated with opioid agonists may be as a result of direct coupling of receptors to adenylyl cyclase via a stimulatory G protein (G_s) (Russell and Potter, 2001). There was evidence to suggest that a subset of opioid receptors may be linked directly to G_s and thereby mediating stimulation by adenylyl cyclase (Cruciani et al., 1993). Thus, it is possible that the opioid receptors couple to both G_i and G_s in B50 cells and the stimulation of adenylyl cyclase by opioid agonists could occur as a result of μ-, δ- and κ-receptors linked to multiple G protein-subunit mediated pathways. The stimulation of adenylyl cyclase resulting in the increase in cAMP has been shown to occur through βγ-subunits of $G_{i/o}$ (Federman et al., 1992), which synergize with G_s to elevate cAMP levels (Mhaouty-Kodja et al., 1997). Newton and Klee, (1990), had observed that the α-subunits of two molecular species of G_i,(G_{i-1} and G_{i-2A}), cause stimulation rather than inhibition of adenylyl cyclase in a bovine brain. Taken together, the results presented from the cAMP experiments in this study suggest that μ-, δ- and κ-opioid receptors can evoke both stimulatory and inhibitory processes on

B50 neuronal cells in culture.

Opioids have been known to modulate cAMP formation in various cells and tissues and their main effect is to inhibit adenylyl cyclase activity and attenuate cAMP formation which is mediated by pertussis toxin-sensitive, Gi-GTP binding proteins. However, some studies have demonstrated stimulatory effects in which opioids induced either inhibition or stimulation depending on the concentration of the drug, the duration of incubation or the presence of forskolin (Rubovitch et al., 2003). In their study Fields and Sarne, (1997), and Sarne et al. (1998), had reported dual effects of opioids on cAMP formation in SK-N-SH neuroblastoma cells in which they showed that at a low concentration, DAMGO stimulated cAMP production, while a higher concentration, inhibited cAMP production. This may be true as in the results presented here in which DAMGO at lower (10μM) concentrations, produced higher cAMP (2.5pmol/ml) accumulation in B50 cells while at higher (100μM) concentrations produced lower cAMP (1.5pmol/ml) accumulation. This is also true in the case of treatment in this study with the kappa opioid agonist (ICI-199441) in which the cAMP release decreased with increase in the concentration of the agonist. Rubovitch et al. (2003), had observed that low concentrations of opioids stimulated cAMP production, while a high concentrations of opioids exert an inhibitory effect, which overcomes and masks the stimulatory pathway. There were similar concentration-dependent dual modulation by opioids in which low concentrations of opioids potentiated, while high concentrations suppressed, the calcium component of the action potential in dorsal root ganglion and visceral neurons (Shen and Crain 1989; Makman et al., 1988). Rubovitch et al. (2003) had shown that the stimulatory effect of DAMGO was mediated by phospholipase C (PLC), and since the most common activator of PLC is Gq-GTP binding protein, it is reasonable to assume the involvement of Gq in the opioid stimulatory pathway. This assumption was supported by the finding that opioid receptors were associated with the stimulatory G_q-GTP binding protein in transfected cells (Kramer and Simon, 1999). Hence there may be two parallel pathways operating within the cells, namely an inhibitory process which is mediated by Gi-GTP binding proteins, and a stimulatory process which is not mediated by PTX-sensitive GTP (G_s and G_q) binding proteins (Rubovitch et al., 2003; Sidhu and Niznik 2000), resulting in elevation of cAMP accumulation in B50 cells treated with opioid agonist in culture. The overall outcome either stimulation or inhibition of cAMP production is dependent on the

balance between the two overlapping pathways. Figure 4.5 show the activation of cAMP pathway leading to cellular induced changes.

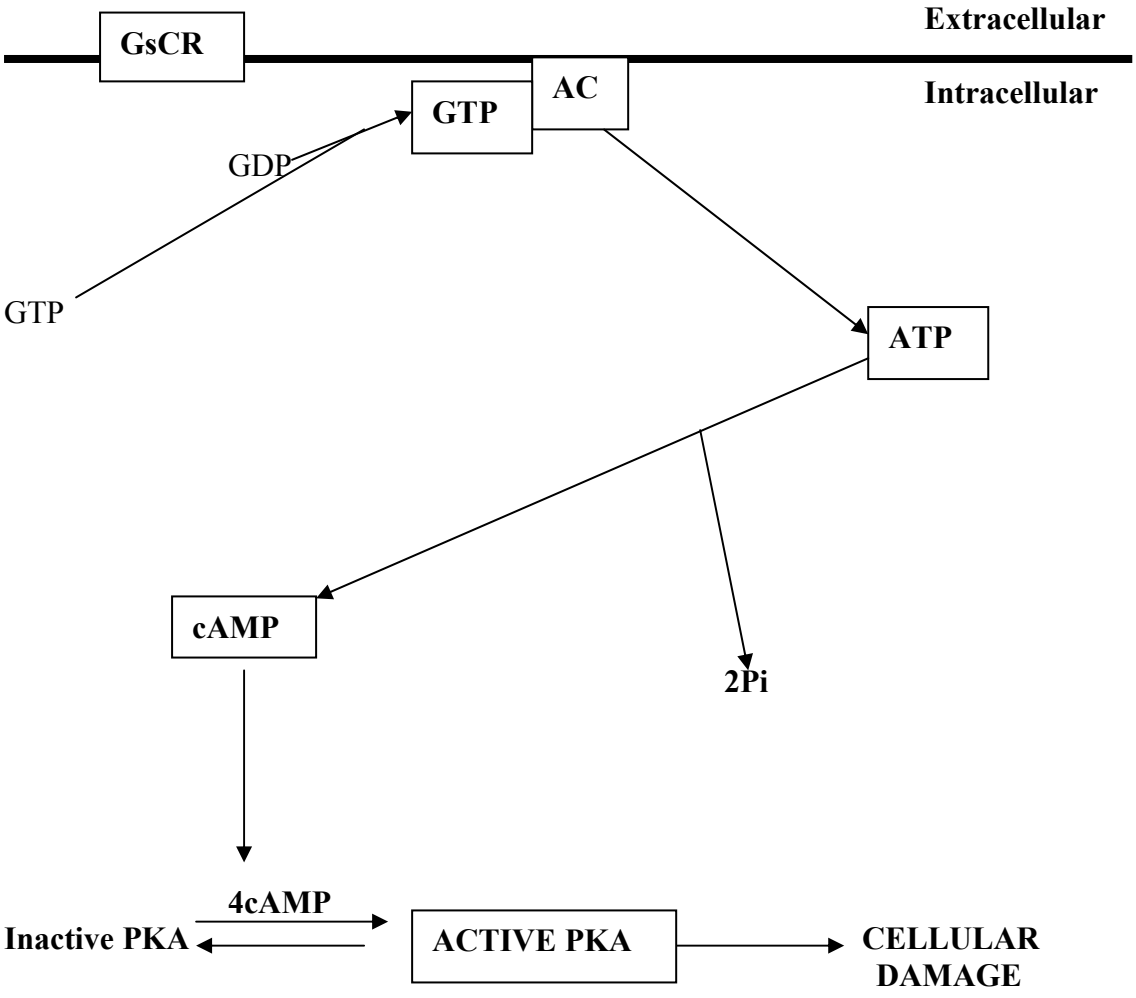


Fig 4.5. Illustration of activation of cAMP pathway leading to cellular induced changes and effects. Adapted from Biocarta (2007).

4.11 Extracellular signal-regulated kinase (ERK) Signalling

The cell based ELISA was used to quantify phospho-ERK1/2 in the study of the effects of hypoxia on ERK pathway activation in the B50 cell line and determine the effect of various cannabinoid and opioid agonist treatments on this pathway. The effects of hypoxia and the treatment with cannabinoid and opioid agonists on phospho-ERK1/2 in B50 cells in culture (Tables 3.20 and 3.21), show that the phospho-ERK1/2 was decreased in untreated hypoxic cells when compared with the normoxic cells. When compared with the hypoxic treated B50 cells, the result show increase in phospho-ERK1/2 in the cells treated with cannabinoid agonists while those treated with opioid agonists showed minor increases which were reversed by the selective cannabinoid and opioid antagonists. The effect of cannabinoid agonist treatment was concentration-dependent. The treatment with Win agonist show that the amount of phospho-ERK1/2 in the treated cells was decreasing with increasing concentration of Win, hence 10nM Win had the highest amount of phospho-ERK1/2 followed by 50nM Win and then 100nM Win had the least amount. The results showed that the treatment of B50 cells with cannabinoid agonists could have affected the cellular production of phospho-ERK1/2 resulting in increased intracellular concentration of phospho-ERK 1/2 when compared with the untreated hypoxic B50 cells in culture. This increase could have resulted in increased downstream cellular activities such as cellular growth, proliferation, differentiation, morphological integrity and activities, and cell death. This is because cannabinoids have been shown to modulate the extracellular signal-regulated kinase (ERK), leading to various forms of plasticity in the brain (Moranta et al., 2007; Daigle et al., 2008). Cannabinoid agonist (Win), increased the immunodensities of phosphorylated ERK1 by 24% and ERK2 by 28% in the rat cerebral frontal cortex and these effects were antagonized by a selective CB_1 antagonist, SR141716A, while pre-treatment with SL327, a MEK1/2 inhibitor, increased the basal phosphorylation of MEK1/2 by 74% and prevented the up-regulation of ERK1/2 by 23-31% induced by Win, which demonstrated that the acute stimulation of CB_1 receptors in the frontal cortex results in the sequential phosphorylation of Raf-MEK-ERK cascade (Moranta et al., 2007). The activation of the phospho-ERK1/2 cascade by cannabinoid agonists involved CB_1 receptors coupled to $G_{i/o}$ GTP-binding proteins since this was blocked by selective cannabinoid antagonists (AM251 and AM630).

ERK1 and ERK2 are serine and threonine kinases expressed broadly in normal tissues and various cell lines. They are activated through the phosphorylation of a threonine and a tyrosine residue and activate a signalling cascade, the downstream effects of which have been linked to the regulation of cell growth and differentiation as well as regulation of the cytoskeleton (Chan et al., 2005). The extracellular signal-regulated kinase (ERK) also known as mitogen activated protein kinase (MAPK) has two isoforms namely, ERK1 known as MAP Kinase1 or P44 MAP kinase and ERK2 known as MAP kinase 2 or P42 kinase. They are activated upon treatment of cells with mitogens, hormones, growth factors, cytokines and bioactive peptides (Chan et al., 2005; Zhuang and Schnellmann, 2006). The results presented here, show that there was an increase in phospho-ERK1/2 in the cannabinoid treated B50 cell groups when compared with the untreated hypoxic groups which potentially could have resulted in increased cellular activities like proliferation and differentiation. This is because the MAP kinases, known as extracellular signal-regulated kinases (ERKs), act as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development (Zhuang and Schnellmann, 2006). The activation of this kinase requires its phosphorylation by upstream kinases such as protein kinase A and protein kinase C. Upon activation, this kinase translocates into the nucleus of the stimulated cells, where it phosphorylates nuclear targets necessary for cellular growth, proliferation, differentiation and normal development (Robinson and Cobb, 1997; Belcheva and Coscia, 2002; Demuth and Molleman, 2006). The increase in phospho-ERK1/2 in cannabinoid agonist treated B50 cells in hypoxia is in agreement with the findings of Derkinderen et al. (2003) and Valjent et al. (2001), in which acute Δ^9-THC administration increased CB_1 receptor-mediated ERK1/2 activation in the dorsal striatum, nucleus accumbens, and hippocampus, while chronic Δ^9-THC treatment has been shown to increase ERK1/2 protein levels in the prefrontal cortex and hippocampus *in vivo* (Rubino et al., 2004).

Ras proteins are small G proteins that are embedded on the inner surface of the plasma membrane (Sebolt-Leopold and Herrera, 2004; Ui, 1994). They are activated by a variety of transmembrane receptors, a process that involves the exchange of GTP for GDP. Ras proteins represent an important signalling branch point because they activate several signalling pathways through a number of effectors such as the protein

kinases of the Raf family (Jo et al., 1997). These cytosolic protein kinases are the first components of a three-tiered protein kinase cascade, which includes two other cytosolic protein kinases namely, MEK and ERK (Dumaz and Marais, 2005; Jo et al., 1997). ERK phosphorylates many substrates, thereby regulating numerous cellular functions, such as gene expression, metabolism and cellular morphology. Consequently ERK signalling plays an important role in determining cell fate, choosing between diverse responses such as proliferation, differentiation, senescence or survival and it also regulates specialist functions such as those in neurons and immune cells (Sebolt-Leopold and Herrera, 2004; Baillie et al., 2000; van Biesen et al., 1996).

The effect of opioid agonist treatment on phospho-ERK1/2 (Table 3.21), shows a slight increase in the quantity of phospho-ERK1/2 in the opioid agonist treated group of hypoxic cells when compared to the untreated hypoxic B50 cells. Additionally, phospho-ERK1/2 was decreased with increasing concentration of the agonist hence, the treatment of B50 cells in hypoxia with the opioid agonist (ICI-199441 and DSLET), 10μM had the highest amount of phospho-ERK1/2 followed by 50μM and 100μM had the least phospho-ERK1/2. The reduction of phospho-ERK1/2 in some treatment groups may be due to high concentration of the agonists which may result in decreased cellular activities and functions as a result of receptor desensitization since increases in the ERK1/2 cascade following chronic drug treatment *in vivo* are most likely to be regulated by receptor desensitization at the cellular level (Derkinderen et al., 2003; Valjent et al., 2001). The results show that since B50 cells are neuronal in origin, the phospho-ERK1/2 activities are more in normal B50 cells when compared to the untreated hypoxic B50 cells which give an indication of normal proliferation, differentiation and growth. These normal neuronal activities was disrupted during hypoxic exposure of the B50 cells resulting in the reduction of the phospho-ERK1/2 activities and the treatment of the hypoxic B50 cells with the agonists brought the phospho-ERK1/2 activities towards the normal levels. Dumaz and Marais (2005), have shown that in neuronal precursor cells, transient ERK signalling stimulates proliferation whereas sustained signalling induces differentiation while in fibroblasts, ERK signalling is essential for proliferation, but high intensity signals induce cell cycle arrest or senescence (Kolch, 2000). Thus ERK has been shown to be a key regulator of cellular behaviour and ERK signalling has long been associated with

cancer where its signalling is disrupted in approximately 30% of cases (Sebolt-Leopold and Herrera, 2004; Dumaz and Marais, 2005, Davis and Laroche, 2006; Davis et al., 2000).

The main mechanism for coupling opioid receptors with the ERK pathway was shown to involve the activation of Ras via Gβγ subunits that are associated with $G_{i/o}$ protein activity (Audet et al., 2005). Other mechanisms by which opioid receptors could regulate the ERK pathway involve the modulation of protein kinase A and C signalling systems (Luttrell and Luttrell, 2003; Belcheva et al., 2005). Acute μ and κ opioid agonists activate the ERK/MAPK phosphorylation cascade that represents an integral part of the signalling pathway of growth factors in astrocytes and by this means, opioids may impact neural development and plasticity among other basic neurobiological processes *in vivo* (Asensio et al., 2005). The μ agonist, (DAMGO), induces a transient stimulation of ERK phosphorylation, whereas κ agonist, (U69,593), engenders sustained ERK activation (Belcheva et al., 2005). There is evidence to demonstrate that acute U69,593 and DAMGO can stimulate ERK phosphorylation by utilizing different secondary messengers and protein kinase C (PKC) isoforms upstream of the growth factor pathway (Belcheva et al., 2005; Yoon and Seger, 2006). The findings relating to the effects of cannabinoid and opioid agonists on phospho-ERK1/2 suggest that differences in their mechanism of signalling may contribute to the outcomes on ERK modulation induced by cannabinoid and opioid agonists as in the differences in cell proliferation and differentiation.

The result showed that the higher the amount of phospho-ERK1/2 in B50 cells, the higher the cellular based activities such as proliferation, differentiation and morphological activities as in pattern formation. The extracellular signal-regulated protein kinases (ERK1/2) have been shown to be essential for normal development and functional plasticity of the central nervous system (Chu et al., 2004; Davis, 1993). The MAPK/ERK kinases-1/2 is emerging as an important regulator of neuronal responses to both functional (in terms of learning and memory) and pathologic (in case of regulated cell death) stimuli. It has been shown that ERK signalling plays a beneficial and neuroprotective role in many systems (Cavanaugh et al., 2001; Ji et al., 2007; Molina-Holgado et al., 2005). Aberrant neuronal expression of phosphorylated form of both ERK1/2 and other MAPKs have been shown in Alzheimer's disease

patients' brain in association with markers of oxidative stress (Chu et al., 2004; Colucci-D'Amato et al., 2003; DeGracia et al., 2007).

The results showed that the treatment of B50 cells in hypoxia with cannabinoid and opioid antagonists (Tables 3.20 and 3.21) were able to bring the level of phospho-ERK1/2 back to untreated hypoxic levels which showed that the antagonist was able to abolish the agonist-induced stimulation of phospho-ERK1/2 production. The result showed that the treatment with the selective cannabinoid (CB_1) and opioid antagonists in hypoxia resulted in the attenuation of the phospho-ERK1/2 signalling resulting in the inhibition of the agonist-induced activation by the antagonists suggesting the involvement of the cannabinoid (CB_1) and opioid receptors. The results also suggest a mechanism involving Ras and beta gamma subunits of G proteins are involved in opioid agonist activation of ERK1/2 as well as opioid modulation of phospho-ERK1/2-induced activity (Belcheva et al., 2005).

The results presented show the quantification of cellular differentiation, proliferation and phospho-ERK1/2 in normal, hypoxic and hypoxic treated B50 cells and the data show that the higher level of phospho-ERK1/2 in B50 cells, correlated with increased cellular differentiation and proliferation indicating the significant involvement of phospho-ERK1/2 in these cellular processes. The results also showed a significant decrease in phospho-ERK1/2 from the normoxic to the hypoxic untreated groups and the treated hypoxic groups, which suggest an improvement in cellular activities in treated hypoxic groups when compared to untreated hypoxic groups.

The results presented (Figures 3.40, 3.42, 3.44 and 3.45), show that the level of cAMP and phospho-ERK1/2 were decreased in the hypoxic untreated B50 cells when compared with the control and those treated with cannabinoid and opioid receptor agonists which may provide a link between the decreased level in cellular proliferation, differentiation and growth and morphological changes in the experimental groups when compared with the control and the hypoxic treated groups. The increased levels of cAMP and phospho-ERK1/2 in the normal B50 may suggest the increased signalling potential in these cells when compared with the hypoxic untreated cells while the treated B50 cells showed increased signalling potential when compared with the hypoxic untreated B50 cells. These signalling activities are in the

form of cellular growth, proliferation and differentiation which are more in the normal B50 cells when compared with the hypoxic untreated and hypoxic treated B50 cells. Dikic and Giordano (2003) had shown that binding of external factors like cannabinoid and opioid agonists to cell membrane receptors, triggers intracellular signalling pathways that ultimately determine if the cell proliferates, differentiates or undergoes apoptosis. Activated receptors also initiate a cascade of events, called negative receptor signalling, that decreases the amplitude of positive signals and modulates the level of cell stimulation. Dikic and Blaukat (1999) had shown that protein tyrosine kinases (PTKs) convey signals from G protein-coupled receptors (GPCRs) to regulate cell proliferation, migration, adhesion, and potentially cellular transformation and death (Subramaniam and Unsicker, 2006). Molecular mechanisms by which PTKs regulate such diverse effects in GPCR signalling are not well understood. It has been shown that both growth factors and GPCRs utilize protein tyrosine kinase activity and the Ras/MAP kinase pathway to control mitogenic signals and activities (Dikic and Blaukat, 1999). Many G protein-coupled receptors (GPCRs) generate signals that control cellular differentiation and growth, including stimulation of Ras family GTPases and activation of MAP kinase pathways (Luttrell and Luttrell, 2003). The mechanisms that these GPCRs use to control the activity of MAP kinases vary between receptor and cell types and these receptors are coupled to the activation of the ERK cascade (Figure 4.6).

The CB_1 receptor has been shown to regulate different members of the MAPK (Bouaboula et al., 1995). Several mechanisms could be responsible for mediating ERK activation by G protein coupled receptors. In PC12 neuronal-like cells, short-term ERK activation promotes proliferation, while sustained ERK activation results in cell cycle arrest and neuronal differentiation (Galve-Roperh et al., 2002). The G protein coupled receptors have been shown to be functionally coupled to inhibition of adenylate cyclase, modulation of ion channels and activation of extracellular-signal-regulated kinase (Galve-Roperh et al., 2002; Cadwallader et al., 1997; Kelly et al., 2003). This kinase plays a pivotal role in the regulation of basic cell functions such as cellular energy metabolism, cellular proliferation and migration (Gomez del Pulgar et al., 2000; Kolch, 2000; Chan et al., 2005). The actions of cannabinoids have been shown to control cell growth and induce proliferative effects in a number of cultured cell models. The ERK/MAPK cascade plays a central role in regulating cellular

proliferation and differentiation. For example, the ability of growth factors to promote and regulate cell growth depends on the activation of receptor tyrosine kinases, which recruit Ras family of small G proteins and leads to sequential activation of Raf family of Kinases. The sequential nature of ERK/MAPK cascade provides multiple points at which the response can be regulated by phosphorylation and dephosphorylation and allows for amplification of extracellular signals (Impey et al., 1999; Bohn et al., 2000; Kolch, 2000). Zhu et al. (2002), have observed cytoplasmic aggregates of phosphorylated extracellular signal-regulated protein kinases in Lewy body diseases.

Dumaz and Marais (2005), have shown that there is an interaction between the cyclic adenosine monophosphate (cAMP) and RAS/RAF/mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways and showed that these two important signalling pathways regulates a large number of cellular functions (Sweatt, 2001). ERK phosphorylation inactivates the enzyme resulting in an increase in the basal levels of cAMP (Hoffmann et al., 1999; Kelly et al., 2003). The results presented in this study suggest an interaction between the cAMP and ERK pathways in the B50 cells in which the effects of the agonists in one pathway may influence the effect in another pathway. This may be the situation in which the cannabinoid agonist (Win) treatment in B50 cells in culture led to increase in both cAMP and phospho-ERK1/2. The mechanisms involved could either be due to the interaction between the cAMP and ERK pathways or the interaction between the endogenous cannabinoid and opioid systems in the cells. Figure 4.6 shows the interaction between the cAMP and ERK signalling pathways.

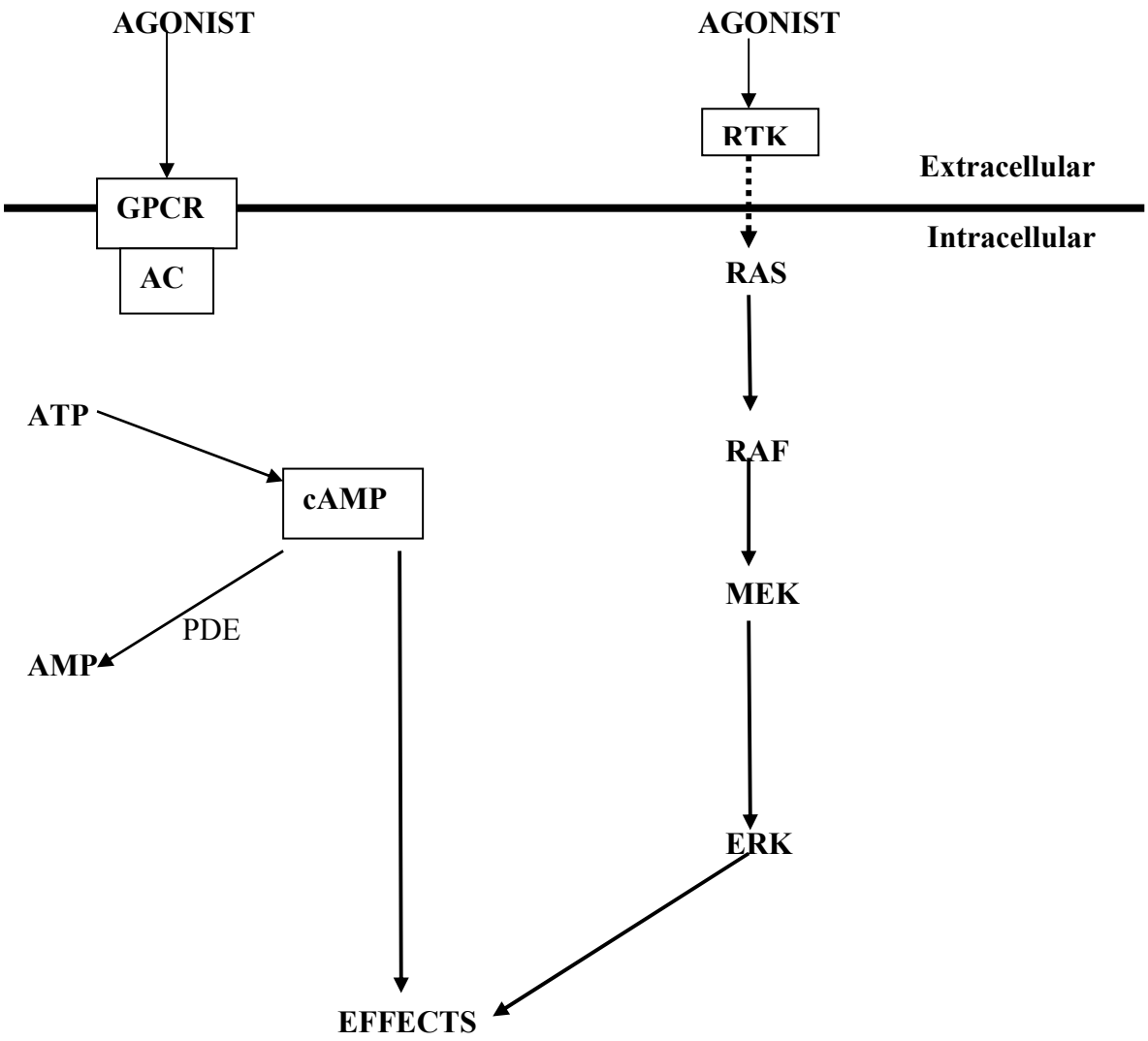


Fig. 4.6. The cAMP and ERK signalling pathways are activated when agonists bind to GPCRs coupled to heterotrimeric G proteins and RTK leading to the activation of small G protein RAS through which ERK is activated. Adapted from Dumaz and Marais (2005).

CHAPTER FIVE

5. Summary and Conclusion

5.1 Summary and Conclusion

The effects of hypoxia on neuronal cell signalling and the neuroprotectant effects of cannabinoid and opioid agonists against hypoxia were investigated using rat cortical neuronal B50 cells in culture. The effects were investigated using different study methods to assess the extent of damage to the cells in hypoxia and if the cell damage could be treated or protected against using cannabinoid and opioid receptor agonists. The methods used include the same field morphological assessment, live cell and death studies using the trypan blue exclusion method, cellular proliferation studies using Promega proliferation assay method, cellular differentiation using DbcAMP and neuronal counting method, cellular death and viability using the lactate dehydrogenase (LDH) assay method and neuronal physiological assessment was done using a quantitative second messenger assessment with cAMP assay and extracellular signal-regulated receptor kinases 1 and 2 (ERK1/2) using enzyme-linked Immunosorbent (ELISA) phospho-ERK1/2 assay methods.

The results from morphological studies showed masses of dead and degenerating neuronal cells resulting from exposure of the B50 cells to hypoxia which could lead to loss of neuronal cell mass and volume. This agrees with the reduced neuronal mass and volume seen in cases of neurodegenerative diseases like stroke, dementias, Alzheimer's and Parkinson's diseases, of which the behavioural effects of these changes depend on the part of the brain tissue where these changes occur. The B50 neuronal cells were derived from the rat cortical neurons, and as a result resemble the cortical neuronal cells in their morphological, biochemical, physiological and behavioural activities and functions as shown from their activities such as differentiation, neurite formation and neurite extension in culture.

These morphological changes are the result of the death and degeneration of the neuronal cells which may result in defects in neuronal functions such as neuronal signal transmission and coordinated response to stimuli through the neuronal synapses due to the loss of neuronal cells in hypoxia. The results presented on the cellular viability, proliferation and differentiation showed that hypoxia affected the functional abilities of these B50 cells in performing their basic cellular and neuronal functions,

and this is consistent with the view that the morphological alterations have led to functional disadvantages in these B50 cells in culture. These cellular losses could account for the cellular and morphological basis of decline in memory function, cognitive and behavioural dysfunction and physical disabilities that accompany different forms of dementias, stroke, Alzheimer's and Parkinson's diseases and other neurodegenerative disorders.

The results on morphological alterations and changes in B50 cells during hypoxia was correlated with the changes observed in the estimated total neuronal cell numbers and density as well as viability, which suggested that the degeneration of the B50 cells in hypoxia has affected the total neuronal numbers and density, and viability of these B50 cells in culture. These morphological and structural changes also resulted in changes in enzyme functions and activities as shown by the changes in lactate dehydrogenase (LDH) enzyme activity, which could have led to the exacerbation of the hypoxic condition and hence may play a role in contributing to the pathologic mechanisms of brain dysfunction in some neurodegenerative disease processes.

The data presented in this study suggest that the selective cannabinoid agonists (Win, AEA and 2-AG) and opioid receptor agonists (DAMGO, DSLET and ICI-199441) may possess some potential therapeutic and protective benefits which could be concentration-dependent in hypoxic situations by rendering B50 cells treated and pre-treated with the cannabinoid and opioid agonists, morphologically healthier when compared with the untreated hypoxic cells. The actions of the cannabinoid agonists were abolished by the treatment with the cannabinoid antagonists (AM251 and AM630), suggesting that the actions of the agonists may be through the cannabinoid receptor and non-receptor mediated. The effects of the opioid agonists were inhibited by the treatment with the selective μ-, δ- and κ-opioid antagonists suggesting the involvement of μ-, δ- and κ-opioid receptors in mediation of these effects in B50 cells in culture.

The morphological and structural alterations were accompanied by significantly elevated mean values of LDH in the B50 cells cultured in hypoxia when compared with the normal and hypoxic treated cells. Hypoxia results in cellular damage and, as such, LDH leaked out of the cells through the membrane into the culture media

leading to the accumulation of the LDH in the media. These alteration in morphology and LDH levels was affected by the treatment with cannabinoid and opioid agonists, which was shown to be concentration-dependent even though that the B50 cells did not seem to react to hypoxic and treatment conditions in the same way. This was shown to be true in the case of the B50 cells in hypoxia but showed normal morphology and consequently normal function. These observations showed a variable vulnerability between the B50 cells during hypoxia and during treatment leading to some of the cells having normal morphology during hypoxia while some even degenerated during the therapeutic treatment which may be as a result of higher concentrations of the cannabinoid and opioid agonists administration.

Two mechanisms acting simultaneously could be responsible for the hypoxic induced cell death and degeneration in B50 cells in culture. These include ATP depletion from the B50 cells in hypoxia and Ca^{2+} excitotoxicity of B50 cells. Since brain cells are extremely sensitive to changes in oxygen and glucose supply, lowering of oxygen supply, as in the hypoxic conditions of the B50 cells in culture, leads to decreased ATP synthesis resulting in depletion of cellular ATP and energy with subsequent death of the neuronal cells. As a result, hypoxia initiates neuronal cell death by removal of the substrate oxygen from cytochrome oxidase making it sensitive to levels of hypoxia. Hypoxic conditions in the brain can be as a result of ischaemia, strokes, trauma or atherosclerosis which could result in the death and degeneration of the neuronal cells.

Neuronal cell death and neurodegenerative processes may also be due to increases in intracellular Ca^{2+} which may subsequently provoke abnormal activation of Ca^{2+}-dependent enzymes such as neuronal nitric oxide synthase (nNOS). In hypoxia, there are increases in extracellular excitatory neurotransmitters and Ca^{2+} which causes excitotoxicity resulting in neuronal cell death. The activation of opioid and cannabinoid receptors may play a role in feedback regulation by hyperpolarization and lowering of the neuronal excitability. It is possible that Ca^{2+} entry may be involved in opioid and cannabinoid receptor upregulation as an early response of neurons to hypoxia. During hypoxia, the cell membrane becomes excited leading to the opening of the ionotropic Ca^{2+} channels resulting in the influx of calcium ions into

the cells. This entry of calcium ions into the cells results in excitotoxicity of the cells and the consequent death and degeneration as the case in hypoxic B50 cells in culture.

The data presented in this study suggest that cannabinoid and opioid receptor agonists are important triggers and mediators of protective and therapeutic response while selective pharmacological antagonists of the cannabinoid and opioid receptor agonists have been shown to block such reactions. The effects of the cannabinoid agonists (Win, AEA and 2-AG), were blocked by the selective cannabinoid antagonists (AM251 and AM630) which suggest that the actions of these cannabinoid agonists were mediated through the CB_1 receptors, through the $G_{i/o}$ proteins and also through a non-receptor mechanism such as ran and ras. while the actions of the opioid agonists (DMAGO, DSLET and ICI-199441), were inhibited by the selective μ-, δ- and κ-opioid antagonists (CTAP, ICI-174864 and NOR), suggesting the involvement of the μ-, δ- and κ-opioid receptors through the inhibitory $G_{i/o}$ proteins in the mediation of the effects of the opioid agonists in B50 cells in culture though there was evidence of some involvement of the stimulatory G_s protein pathway. This stimulatory pathway was observed at some high concentrations of both cannabinoid and opioid agonists resulting in the opposite effects of the inhibitory pathway. The actions of the agonists and antagonists were shown to be concentration-dependent since different levels of antagonists were required to counter the action of the agonists.

The results show that there were some differences in the action of the agonists used in this study which could be due to the effects of the interaction between the agonists and other endogenous agonists, and between the agonists and the other receptor types present in the B50 cells. This is because the cannabinoid agonists (Win, AEA and 2-AG), used in this study could be interacting with other endogenous cannabinoids that are normally present in the cells thus leading to the augmentation or exacerbation of the effects in this experimental model. Cannabinoid agonists could also be interacting with other non-cannabinoid ($CB_{1/2}$) receptor targets such as opioid receptors, orphan G protein-coupled receptors (GPR55 and GPR119), vanilloid receptors (TRPV1) and peroxisome proliferator-activated receptors (PPARs). These non-$CB_{1/2}$ receptors have been shown to contribute to behavioural, vascular, and immunological actions of Δ^9-THC and endogenous cannabinoids (Lauckner et al., 2008). The GPR55 is a cannabinoid receptor that is highly expressed in large dorsal root ganglion neurons

and upon activation by various synthetic and endogenous cannabinoids, increases intracellular calcium in these neurons through G_q, G_{12}, G_{13}, GTPγS binding, phospholipase C and calcium release from IP_3R-gated stores, indicating that GPR55 as a cannabinoid receptor with signalling distinct from the $CB_{1/2}$ (Lauckner et al., 2008; Pertwee, 2007, Ryberg et al., 2007). The TRPV1 receptor is activated by noxious stimuli including heat, hydrogen ions and capsaicin and can be found on sensory neurons, where TRPV1 channel opening causes Ca^{2+} influx and neurotransmitter release (Demuth and Molleman, 2006). It has been shown that anandamide can activate the TRPV1 receptor, although it is thought to do so by binding to sites on the cytosolic side of the receptor indicating that anandamide could activate TRPV1 channels in physiological conditions. Anandamide has also been shown to activate TRPV1 receptors in rat hippocampal slices and have dual effects on inhibitory CB_1 and excitatory TRPV1 receptors (Demuth and Molleman, 2006). The PPARs are a group of nuclear receptor proteins that function as transcription factors regulating gene expression and they play essential roles in the regulation of cellular differentiation, development and metabolism (Michalik et al., 2006). The PPARs can be identified as α, γ or δ(β) and are expressed in different tissues but δ(β) are markedly expressed in the brain and skin (Balakumar et al., 2007). These non-cannabinoid ($CB_{1/2}$) receptors could be activated in B50 neuronal cells cultured in hypoxia, resulting in some of the observed effects.

Some of the effects observed in this study could result from the pharmacological interactions between the cannabinoid and opioid systems because, it has been suggested that the antinociceptive effects of morphine mediated by μ-opioid receptors, might be enhanced by Δ^9-THC through activation of κ- and δ-opioid receptors (Pugh et al., 1996). Cannabinoids and opioids might interact at the level of their signal-transduction mechanisms (Manzanares et al., 1999), since opioid and cannabinoid receptors are coupled to similar intracellular signalling systems such as the inhibition of adenylyl cyclase activity and Ca^{2+} currents through the activation of $G_{i/o}$ proteins. *In vitro* or *in vivo* exposure to opioid and/or cannabinoid agonists may cause a desensitisation and cross-desensitisation in the ability of the drugs to inhibit forskolin-induced cAMP production (Demuth and Molleman, 2006). Cannabinoids may have a direct effect on the synthesis and release of endogenous opioids. Valverde et al. (2001), showed that acute administration of Δ^9-THC increased the release of

enkephalin-like material in the nucleus accumbens of awake and freely moving rats suggesting cannabinoids can increase opioid release.

Since cannabinoid and opioid receptor agonists may serve as a general protector against ischaemic and hypoxic stress in oxygen and energy-sensitive organs like the brain and heart, their effects on the B50 cells could be due to the anti-inflammatory properties of cannabinoids. This is because cannabinoids have been shown to possess anti-inflammatory properties, which may be relevant in neuroprotection in which cannabinoid agonists could have down-regulated inflammatory cytokines and up-regulated anti-inflammatory ones in neuronal B50 cells cultured in hypoxia.

The study have shown that B50 cells are good model cell line to study the effects of hypoxia on neuronal cell signalling using such downstream signalling activities as cellular morphology, viability, growth, proliferation, differentiation and second messenger cAMP and ERK1/2 activities and showed that these cells could be used to study neuronal cellular signalling in culture since they are derived from cortical neuronal cells. The results presented showed that cannabinoid and opioid receptor agonists have some positive effects on the B50 neuronal cells cultured in hypoxia by making the cells treated with cannabinoid and opioid agonists have healthier morphology, down regulation of LDH release, upregulation of intracellular cAMP and phospho-ERK1/2 when compared with the hypoxic untreated B50 cells in culture and as such, the signalling activities like cellular proliferation, differentiation, second messenger cAMP and ERK1/2 activities are shown to improve in the hypoxic treated B50 cells than in untreated hypoxic cells. The summary diagram showing the CB_1 and opioid receptors through the signalling pathways involved in cell survival, growth, proliferation and differentiation is shown in Figure 5.1. Thus the study have shown that cannabinoid and opioid receptor agonists could have some positive potential benefits in the treatment and protection of hypoxia-related neurodegenerative disorders and diseases such as stroke, dementias, ageing, Alzheimer's and Parkinson's diseases and cognitive decline.

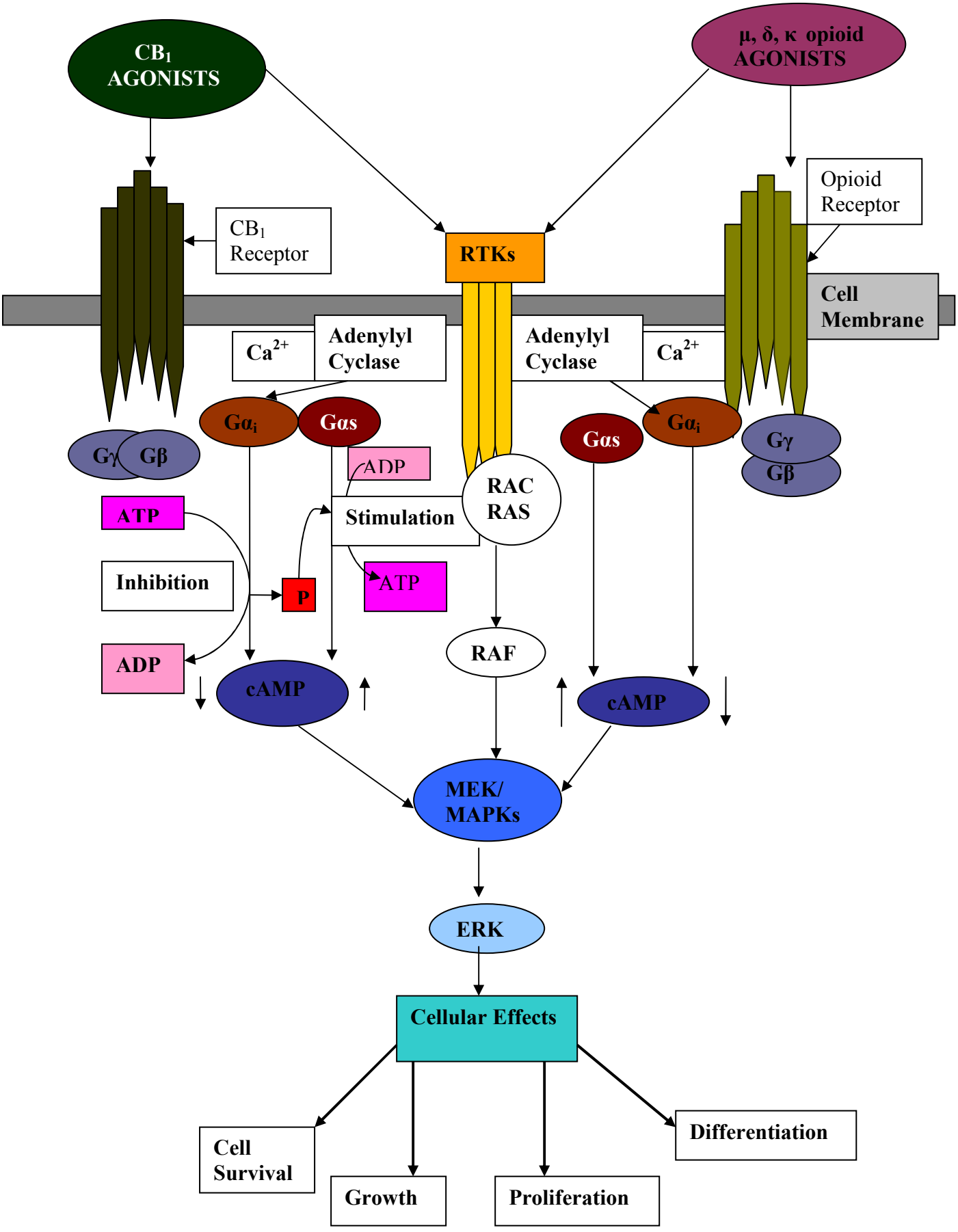

CB1 AGONISTS
μ, δ, κ opioid AGONISTS
CB1 Receptor
RTKs
Opioid Receptor
Cell Membrane
Ca2+
Adenylyl Cyclase
Adenylyl Cyclase
Ca2+
Gαi
Gαs
Gαs
Gαi
Gγ
Gβ
Gγ
Gβ
ADP
ATP
Stimulation
RAC
RAS
Inhibition
P
ATP
ADP
cAMP
RAF
cAMP
MEK/
MAPKs
ERK
Cellular Effects
Cell Survival
Growth
Proliferation
Differentiation

Fig.5.1.Summary diagram of the CB_1 and μ, δ and κ opioid receptor activation and the signalling pathways involved in cell survival, growth, proliferation and differentiation in B50 cells cultured in hypoxia. The CB_1 and μ-, δ- and κ-opioid receptors are activated by the binding of the agonists to their receptors, leading to the dissociation of G-protein Gαβγ complex into Gα and Gβγ proteins and the activation of adenylyl cyclase which results in the phosphorylation of ADP to ATP and the subsequent conversion of ATP to cAMP, the secondary messenger which act through many effectors to produce cellular effects through the inhibitory pathway of $G\alpha_{i/o}$ leading to the inhibition of cAMP accumulation within the B50 cells resulting in decrease in cAMP, while some can act through the stimulatory pathway of $G\alpha_s$ to stimulate cAMP accumulation within the cells leading to increase in cAMP accumulation. These agonists through their receptors activate receptor tyrosine kinase, an enzyme found around these receptors, resulting in the activation of small G proteins RAS and RAC which subsequently activate the RAF/MEK/ERK signal transduction pathway which is a conserved pathway that regulates cell growth, proliferation and differentiation. These activated proteins stimulate constitutive signalling through the pathway in the B50 cells to stimulate growth, proliferation and differentiation of the cells and protect them from cell death. Essentially these kinases are normally trapped in inactive forms but through the intramolecular interaction of the agonists and the receptors, are released into the active form. The activation of the receptors by the agonists resulting in the activation of the stimulatory pathway can also lead to the reduction of calcium influx into the cells and reduce ATP consumption leading to the conservation of cellular energy needed for other activities within the cells such as growth and differentiation.

5.2 Limitations of the study

There were some limitations encountered during the study of the effect of hypoxia on neuronal cell signalling in B50 cells in culture. Since the B50 cells are adherent cells and are detached from the floor of the culture plate using trypsin-EDTA solution, this may result in the death of some of the cells due to the oxidizing action of the trypsin-EDTA reagent. Some of the dead cells may be floating in the culture medium and may be lost during the process of decanting the media before the trypan blue exclusion method of estimation of the number of viable and dead cells in culture thus the number of dead cells may be under-estimated. The use of manual counting method may also present with some errors in the overall number of cells counted in that some cells may be missed during counting as in total cell count, total viable cells and number of differentiated cells in culture. There was also the tendency that the use of a colorimetric method as in CellTitre Aqueous cell proliferation assays for the determination of viable cells in proliferation in B50 cells may be prone to some errors. The use of a small amount of culture medium and the CellTitre Aqueous cell proliferation assay reagent may not give the overall effect on cellular proliferation on B50 cells in culture. This is because the factors that affect the metabolic activity of the cells may affect the relationship between cell numbers and absorbance and since B50 cells are adherent, cells may undergo contact inhibition which may show a change in metabolic activity at high densities, resulting in a nonlinear relationship between cell numbers and absorbance since the factors that affect the cytoplasmic physiology of the cells will affect metabolic activity.

The study on LDH release may be subjected to some limitations in that the culture medium contained serum, though heat-inactivated which may have some level of LDH activity since the use of medium containing serum could result in some levels of LDH to decrease the sensitivity of the assay or obscure the detection of cellular LDH completely. This was corrected by the measurement of the optical density of the culture medium. Also the reduced nicotinamide adenine dinucleotide (NADH) formed in the process of the assay is subject to photo degradation and as such any accidental exposure of the reaction to light may reduce the sensitivity and accuracy of the LDH assay results in this study. The culture medium may be subjected to uneven evaporation from the multiwells which may cause erroneous results. The reconstituted cofactor preparation and storage at higher temperatures may result in loss of activity

and yield erroneous results. The cAMP concentration assays were measured from B50 cell culture media and were diluted with 0.1M HCl which may tend to dilute the cAMP concentrations and also there may be high levels of endogenous cAMP which may interfere with the results. Limitations to the procedure of cAMP assay method may result in variations in cAMP assay data in that there may be fluctuations in optical density readings during the shelf-life of the assay kit, even though the results are still comparable.

The possibility that the phospho-ERK1/2 present in B50 cells in this study may be degraded by the cell extraction buffer used, presents a limitation of this procedure although it has not been described before. The phospho-ERK1/2 assay showed that only phosphopeptides containing the phosphorylated threonine 185 and tyrosine 187 blocked the ELISA signal but the same sequence containing non-phosphorylated threonine and tyrosine at position 185/187 or mono-phosphopeptides may also produce a small blockage of the ELISA signal which may be a limitation to the study.
Above all, funding and unavailability of some research equipments also had been a source of limitations to this study in that this research did not have any special funding on its own but rely mainly on the departmental funds and donation of reagents from companies. Some equipments were not available to enable some specific investigations such as calcium ion capture to measure intracellular calcium contents in the cells in culture and the agonists and membrane binding assay equipment for comparative receptor binding analyses of cannabinoid and opioid agonists and antagonists.

5.3 Research Applications

The findings of this research have several implications and potential applications which could be of interest to government and agencies, research institutes and research charities that are involved in neurodegenerative changes, their treatment, prevention and protection. The findings can be of interest and applied in the following ways:

- The B50 neuronal cells which were derived from neonatal cortical cells have been shown to grow very well in both normoxic and hypoxic environments and also have been shown to develop, proliferate and differentiate very well in

culture environment and as such a good research model for the study of neuronal cells in culture.

- Since the B50 cells are neonatal cells that can grow and develop well into normal neuronal patterns in culture, they could be a good source of research cells for stem cell therapy research against neurodegenerative diseases and disorders.
- Also since the B50 cells showed evidence of the presence of G protein coupled receptor most especially the cannabinoid (CB_1) and μ-opioid receptors, it could be a good material for research in areas of agonists-induced and activated effects.
- In the treatment of neurodegenerative diseases and disorders such as stroke, various dementias, Parkinson's and Alzheimer's diseases, the cannabinoid and opioid agonists have shown to have the potential to improve the brain morphology, chemistry and functions and by so doing improve the overall brain activity like learning, memory and cognitive functions and abilities.
- In cases of pre-treatment against hypoxia with cannabinoid and opioid agonists for the prevention and protection against vascular dementia caused by decreased oxygen and nutrient supply due to the occlusion of blood vessels to the brain.
- The cannabinoid and opioid agonists could be useful in the prevention and protection against respiratory distress syndrome in children caused by limiting oxygen supply to the brain in which they could have the potential to improve brain morphology and activities.
- The cannabinoid and opioid agonists could be used for the treatment and protection against sleep apnea-induced brain hypoxia in both adults and children in which these drugs could have the potential to improve the effects on the brain cells.
- The cannabinoid and opioid agonists could have the potential for the prevention and protection of individuals with high stroke risk especially those with prior minor or primary stroke with the possibility of secondary normally massive attack in order to prevent and protect against the secondary massive attack.

- The cannabinoid and opioid agonists could have the potential for the prevention and protection for individuals involved in high altitude activity to prevent and protect them against the effects of low oxygen level exposure. Examples include space travellers, mountain climbers and military personals.

5.4. Future directions

In future studies, it would be worthy to measure and quantify ATP production in B50 cells and normalized to the total number of viable cells, hence be able to determine whether ATP production is affected by hypoxia and the subsequent effect by the cannabinoid and opioid agonists used in the study. It would be necessary in the future to quantify the intracellular calcium ions using fluorescent calcium-sensitive dye, to monitor and measure calcium influx and efflux from B50 cells in normal, hypoxic and hypoxic treated B50 cells in culture. It would be good in the future to measure and quantify the level of oxidative stress in B50 cells using thiobarbituric acid reactive substances (TBARS) or glutathione assays in hypoxic cells and those treated with the agonists.

Since the ultimate goal of any basic laboratory research is to help in alleviating human suffering caused by diseases and illnesses which will lead to successful treatment or prevention of these diseases and illnesses. This research was intended to identify the effects of hypoxia on neuronal cell signalling using cellular based activities as proliferation, differentiation, cell growth and viability, and cell death study using LDH release and the effects produced by the administration of cannabinoid and opioid agonists in the treatment and protection against the effects produced by hypoxia in cortical B50 neuronal cells in culture. In the future, effort would also be made to use animal models, most especially rodents (rats and mice), to conduct a whole animal study using an animal model of hypoxic diseases or animals induced with hypoxia to study the actions of these agonists in the reversal of the effects of hypoxia and the neuroprotective abilities of these agonists in these animal models against hypoxia, which would eventually lead in the testing of these agents in humans to see how these therapeutic and protective potentials could be harnessed for immense medical benefit.

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APPENDICES

Table A1: The effect of opioid agonist treatment on LDH release in cultured B50 cells in hypoxia

Type of Treatment	Measured optical density(OD)	Percent of control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	1.214±0.511	587±51.1*
DAMGO(μ)10μM	0.699±0.421	338±42.1*#
DAMGO(μ)50μM	0.344±0.210	167±21.0#
DAMGO(μ)100μM	0.296±0.112	143±11.2#
DSLET(δ) 10μM	0.346±0.201	167±20.1#
DSLET(δ) 50μM	0.290±0.112	140±11.2#
DSLET(δ) 100μM	0.296±0.221	143±22.1#
ICI-199441(κ) 10μM	0.234±0.221	113±22.1#
ICI-199441(κ) 50μM	0.224±0.223	109±22.3#
ICI-99441(κ)100μM	0.666±0.251	322±25.1*#

(Data presented as means ±SD; n=6; *P<0.05 versus normal B50 cells; #P<0.05 versus hypoxia untreated cells; Student's t-test).

Table A2: The effect of opioid agonist/antagonist treatment on LDH release from cultured B50 cell in hypoxia

Type of Treatment	Measured optical density(OD)	Percent of control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	1.214±0.511	587±51.1*
DAMGO(μ)10μM	0.699±0.421	338±42.1*
DAMGO/CTAP10μM	0.726±0.335	351±33.5*
DAMGO(μ)50μM	0.344±0.210	167±21.0
DAMGO/CTAP50μM	0.535±0.252	259±25.2*#
DAMGO(μ)100μM	0.296±0.112	143±11.2
DAMGO/CTAP100μM	0.752±0.305	364±30.5*#
DSLET(δ) 10μM	0.346±0.201	167±20.1
DSLET/ICI-174864 10 μM	0.530±0.256	256±25.6*#
DSLET(δ) 50μM	0.290±0.112	140±11.2
DSLET/ICI-174864 50 μM	0.859±0.345	416±34.5*#
DSLET(δ) 100μM	0.296±0.221	143±22.1
DSLET/ICI-174864 100 μM	0.644±0.225	312±22.5*#
ICI-199441(κ) 10μM	0.234±0.221	113±22.1
ICI-199441/NOR 10μM	1.698±0.501	821±50.1*#
ICI-199441(κ) 50μM	0.224±0.223	109±22.3
ICI-199441/NOR 50 μM	1.093±0.552	529±55.2*#
ICI-99441(κ)100μM	0.666±0.251	322±25.1
ICI-199441/NOR 100 μM	1.375±0.446	665±44.6*#

(Data presented as means ±SD; n=6; *P<0.05 versus normal B50 cells; #P<0.05 versus agonist treated hypoxic cells; Student's t-test).

Table A3: The effect of opioid agonist pre-treatment on LDH release in cultured B50 cells in hypoxia

Type of Treatment	Measured optical density(OD)	Percent of Control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	1.214±0.511	587±51.1*
DAMGO(μ)10μM	0.878±0.354	425±35.4*#
DAMGO(μ)50μM	0.728±0.321	352±32.1*#
DAMGO(μ)100μM	0.733±0.331	355±33.1*#
DSLET(δ) 10μM	1.076±0.511	520±51.1*
DSLET(δ) 50μM	0.700±0.433	339±43.3*#
DSLET(δ) 100μM	0.930±0.322	450±32.2*#
ICI-199441(κ) 10μM	0.716±0.442	346±44.2*#
ICI-199441(κ) 50μM	0.866±0.544	419±54.4*#
ICI-99441(κ)100μM	0.657±0.342	318±34.2*#

(Data presented as means ±SD; n=6; *P<0.05 versus normal B50 cells; #P<0.05 versus hypoxia untreated cells; Student's t-test).

Table A4: The effect of opioid agonist/antagonist pre-treatment on LDH release in cultured B50 cell in hypoxia.

Type of Treatment	Measured optical density(OD)	Percent of Control (% of Control)
Normal Culture	0.207±0.101	100±10.1
Hypoxia no Drug	1.214±0.511	587±51.1*
DAMGO(μ)10μM	0.878±0.354	425±35.4*
DAMGO/CTAP 10μM	0.486±0.312	235±31.2*
DAMGO(μ)50μM	0.728±0.321	352±32.1*
DAMGO/CTAP 50μM	1.427±0.445	690±44.5*#
DAMGO(μ)100μM	0.733±0.331	355±33.1*
DAMGO/CTAP 100μM	1.268±0.502	613±50.2*#
DSLET(δ) 10μM	1.076±0.511	520±51.1*
DSLET/ICI-174864 10μM	0.800±0.445	387±44.5*
DSLET(δ) 50μM	0.700±0.433	339±43.3*
DSLET/ICI-174864 50μM	1.264±0.421	611±42.1*#
DSLET(δ) 100μM	0.930±0.322	450±32.2*
DSLET/ICI-174864 100μM	0.607±0.352	294±35.2*
ICI-199441(κ) 10μM	0.716±0.442	346±44.2*
ICI-199441/NOR 10μM	0.342±0.252	165±25.2*
ICI-199441(κ) 50μM	0.866±0.544	419±54.4*
ICI-199441/NOR 50μM	1.279±0.511	619±51.1*#
ICI-199441(κ) 50μM	0.657±0.342	318±34.2*
ICI-199441/NOR 100μM	0.738±0.462	357±46.2*#

(Data presented as means ±SD; n=6; *P<0.05 versus normal B50 cells; #P<0.05 versus agonist treated hypoxic cells; Student's t-test).

Table A5: The effect of opioid agonist treatment on LDH release from normal cultured B50 cells

Type of Treatment	Measured optical density(OD)	Percent of Control (% of Control)
Normal no Drug	0.172±0.122	100±12.2
DAMGO(μ)10μM	0.165±0.108	96±10.8
DAMGO(μ)50μM	0.215±0.191	125±19.1*
DAMGO(μ)100μM	0.183±0.178	107±17.8
DSLET(δ) 10μM	0.137±0.133	80±13.3*
DSLET(δ) 50μM	0.138±0.115	80±11.5*
DSLET(δ) 100μM	0.149±0.121	87±12.1*
ICI-199441(κ)10μM	0.176±0.119	103±11.9
ICI-199441(κ) 50μM	0.139±0.111	81±11.1*
ICI-199441(κ)100μM	0.158±0.122	92±12.2

(Data presented as means ±SD; n=6;*P<0.05 versus untreated normal B50 cells Student's t-test).

Table A6:The effect of opioid pre-treatment on LDH release in normal B50 cells.

Type of Treatment	Measured optical density(OD)	Percent of Control (% of Control)
Normal no Drug	0.172±0.122	100±12.2
DAMGO(μ)10μM	0.165±0.125	96±12.5
DAMGO(μ)50μM	0.183±0.221	107±22.1
DAMGO(μ)100μM	0.224±0.121	131±12.1*
DSLET(δ) 10μM	0.146±0.111	85±11.1*
DSLET(δ) 50μM	0.128±0.101	75±10.1*
DSLET(δ) 100μM	0.120±0.110	70±11.0*
ICI-199441(κ) 10μM	0.099±0.091	58±9.1*
ICI-199441(κ) 50μM	0.116±0.104	68±10.4*
ICI-199441(κ)100μM	0.140±0.115	82±11.5*

(Data presented as means ±SD; n=6; *P<0.05 versus untreated normal B50 cells; Student's t-test).

Table A7: The effect of DbcAMP and cannabinoid agonist treatment on LDH release in normal cultured B50 cells.

Type of Treatment	Measured optical density(OD)	Percent of Control (% of Control)
Normal Culture no drug treatment	0.108±0.105	100±10.5
Cells with DbcAMP only	0.120±0.100	110±10.0
DbcAMP/10nM Win	0.117±0.101	108±10.1
DbcAMP/50nM Win	0.128±0.110	118±11.0
DbcAMP/100nM Win	0.146±0.121	134±12.1

(Data presented as means ±SD; n=6; Student's t-test).

Table A8: The effect of DbcAMP and cannabinoid agonist pre-treatment on LDH release in normal cultured B50 cells.

Type of Treatment	Measured Optical Density(OD)	Percent of Control (% of Control)
Normal Culture no drug treatment	0.108±0.105	100±10.5
Cells with DbcAMP only	0.120±0.100	110±10.0
DbcAMP/10nM Win	0.118±0.101	109±10.1
DbcAMP/50nM Win	0.190±0.121	175±12.1*$^{\#}$
DbcAMP/100nM Win	0.203±0.225	188±22.5*$^{\#}$

(Data presented as means ±SD; n=6; *P<0.05 versus untreated normal; $^{\#}$P<0.05 versus cells with DbcAMP only; Student's t-test).

Table A9: The effect of DbcAMP and cannabinoid agonist ± antagonist on LDH release in normal B50 cells.

Type of Treatment	Measured optical density(OD)	Percent of Control (% of Control)
Normal cells no drug treatment	0.108±0.105	100±10.5
DbcAMP only	0.120±0.100	110±10.0
DbcAMP/10nM Win	0.118±0.101	109±10.1
AM251/10nM Win	0.116±0.102	107±10.2
AM251/50nM Win	0.141±0.121	130±12.1*
AM251/100nM Win	0.207±0.181	191±18.1*#

(Data presented as means ±SD; n=6; *P<0.05 versus untreated normal;
#P<0.05 versus cells with DbcAMP only; Student's t-test).

Table A10: The effect of DbcAMP and cannabinoid agonist treatment on LDH release in B50 cells in hypoxia.

Type of Treatment	Measured optical density(OD)	Percent of Control (% of Control)
Normal Culture no drug treatment	0.108±0.105	100±10.5
Hypoxia no Drug	0.172±0.107	158±10.7*
Hypoxia/DbcAMP	0.206±0.166	190±16.6*#
DbcAMP/10nM Win	0.144±0.122	133±12.2
DbcAMP/50nM Win	0.148±0.119	136±11.9
DbcAMP/100nM Win	0.153±0.131	141±13.1*#

(Data presented as means ±SD; n=6;*P<0.05 versus untreated normal cells;
#P<0.05 versus hypoxia untreated cells; Student's t-test).

Table A11: The effect of DbcAMP and cannabinoid agonist pre-treatment on LDH release in B50 cells in hypoxia.

Type of Treatment	Measured optical density(OD)	Percent of control (% of Control)
Normal Culture no drug treatment	0.108±0.105	100±10.5
Hypoxia no Drug	0.172±0.107	158±10.7*
Hypoxia/DbcAMP	0.206±0.166	190±16.6*#
DbcAMP/10nM Win	0.158±0.122	146±12.2*
DbcAMP/50nM Win	0.198±0.151	183±15.1*#
DbcAMP/100nM Win	0.207±0.156	191±15.6*#

(Data presented as means ±SD; n=6; *P<0.05 versus untreated normal cells; #P<0.05 versus untreated hypoxic cells; Student's t-test).

Table A12: The effect of DbcAMP and cannabinoid agonist/antagonist treatment in B50 cells in hypoxia

Type of Treatment	Measured Optical Density(OD)	Percent of control (% of Control)
Normal Culture no drug treatment	0.108±0.105	100±10.5
Hypoxia no Drug	0.172±0.107	158±10.7*
Hypoxia/DbcAMP	0.206±0.166	190±16.6*#
DbcAMP/10nMWin	0.158±0.122	146±12.2*
AM251/10nM Win	0.197±0.131	182±13.1*#
DbcAMP/50nMWin	0.198±0.151	183±15.1*#
AM251/50nM Win	0.142±0.122	131±12.2
DbcAMP/100nMWin	0.207±0.156	191±15.6*#
AM251/100nM Win	0.231±0.125	213±12.5*#

(Data presented as means ±SD; n=6; *P<0.05 versus untreated normal cells; #P<0.05 versus hypoxia untreated cells; Student's t-test).

Table A13: The effect of opioid agonist treatment on cellular proliferation in normal cultured B50 cells.

Type of Treatment	Measured optical density	Calculated Value $(x10^6)$/ml
Normal Cells no Drug	1.5	20.00±0.47
10uM DAMGO	1.0	15.00±0.30*
100uM DAMGO	1.3	17.50±0.11
10uM DSLET	1.3	17.50±0.53
100uM DSLET	0.88	13.75±0.10*
10uM ICI-199441	1.19	16.88±0.10
100uM ICI-199441	1.56	20.20±0.15

(Data presented as means ±SD; n=6; *P<0.05 versus untreated normal cells; Student's t-test).

Table A14: The effect of opioid agonist treatment on cellular proliferation in B50 cells in hypoxia.

Type of Treatment	Measured optical density	Calculated Value $(x10^6)$/ml
Normal Cells no Drug	1.5	20.00±0.47$^{\#}$
Hypoxic cells no drug	0.35	7.00±0.12*
10uM DAMGO	1.06	16.25±0.53
100uM DAMGO	1.51	20.00±0.54$^{\#}$
10uM DSLET	1.52	20.00±0.70$^{\#}$
100uM DSLET	1.32	17.60±0.27$^{\#}$
10uM ICI-199441	1.36	17.70±0.34$^{\#}$
100uM ICI-199441	1.6	21.52±0.45$^{\#}$

(Data presented as means ±SD; n=6;*P<0.05 versus untreated normal cells; $^{\#}$P<0.05 versus untreated hypoxic cells; Student's t-test).

Table A15: The effect of opioid agonist pre-treatment on cellular proliferation in normal B50 cells.

Type of Treatment	Measured optical density	Calculated Value $(x10^6)$/ml
Normal Cells no Drug	1.50	20.00±0.47
10uM DAMGO	1.51	20.00±0.06
50uM DAMGO	1.57	20.80±0.64
100uM DAMGO	2.00	24.75±0.47
10uM DSLET	1.30	17.50±0.93
50uM DSLET	1.68	15.70±0.77
100uM DSLET	1.47	19.80±0.35
10uM ICI-199441	1.20	17.10±0.51
50uM ICI-199441	1.60	22.40±0.65
100uM ICI-199441	0.90	14.50±0.66*

(Data presented as means ±SD; n=6; *P<0.05 versus untreated normal cells; Student's t-test).

Table A16: The effect of opioid agonist pre-treatment on cellular proliferation in B50 cells in hypoxia.

Type of Treatment	Measured optical density	Calculated Value ($x10^6$)/ml
Normal Cells no Drug	1.5	20.00±0.47
Hypoxic cells no drug	0.35	07.00±0.12*
10uM DAMGO	0.64	11.25±0.02*#
50uM DAMGO	0.70	12.00±0.53*#
100uM DAMGO	0.49	09.75±0.17*#
10uM DSLET	0.37	07.40±0.12*
50uM DSLET	0.89	13.75±0.21*#
100uM DSLET	0.74	12.50±0.37*#
10uM ICI-199441	0.44	09.45±0.15*
50uM ICI-199441	0.45	09.50±0.02*
100uM ICI-199441	0.42	09.40±0.26*

(Data presented as means ±SD; n=6;*P<0.05 versus normal cells; #P<0.05 versus untreated hypoxic cells; Student's t-test).

Table A17: The effect of opioid agonist/antagonist treatment on cellular proliferation in B50 cells in hypoxia.

Type of Treatment	Measured optical density	Calculated Value ($x10^6$)/ml
Normal Cells no Drug	1.5	20.00±0.47
Hypoxic cells no drug	0.35	07.00±0.12
50uM DAMGO	0.70	12.00±0.53*
DAMGO/CTAP 50µM	1.43	18.50±0.63*#
100uM DAMGO	0.49	09.75±0.17
DAMGO/CTAP 100µM	1.56	20.10±0.83*#
50uM DSLET	0.37	07.40±0.12
DSLET/ICI-174864 50µM	1.12	16.25±0.37*#
100uM DSLET	0.74	12.50±0.37*
DSLET/ICI-178464 100µM	0.96	14.90±0.10*
50uM ICI-199441	0.45	09.50±0.02
ICI-199441/NOR 50µM	1.38	18.75±0.68*#
100uM ICI-199441	0.42	09.40±0.26
ICI-199441/NOR 100µM	1.28	17.50±0.42*#

(Data presented as means ±SD; n=6;*P<0.05 versus untreated hypoxic cells; #P<0.05 versus agonist treated hypoxic cells; Student's t-test).

Table A18:The effect of hypoxia on B50 cell differentiation using DbcAMP and random field assessment method.

Type of treatment	Mean number of differentiated cells
Normal	56.44±13.50
Hypoxia	28.17±10.70*
Normal/Hypoxia	19.72±7.27*#

(Data presented as means ±SD; n=6;*P<0.05 versus normal cells;
#P<0.05 versus untreated hypoxic cells; Student's t-test).

Table A19: Standard table of absorbance versus cell concentration used in standard curve in cell proliferation assay in time-course effect of hypoxia.

Dilution	Absorbance (490nm)	Cell concentration (10^6)/ml
0	3.73±0.57	46.5
1:2 (50%)	1.67±0.30	23.25
1:4 (25%)	0.52±0.18	11.63
1:5 (20%)	0.38±0.26	9.30
1:10(10%)	0.20±0.16	4.65

(Data presented as mean ±SD; n=5)

Table A20: Standard table of absorbance versus cell concentration used in standard curve in cell proliferation assay in agonist treated cells in hypoxia.

Dilution	Absorbance (490nm)	Cell concentration (10^6)/ml
0	2.71±0.76	42.50
1:2 (50%)	1.29±0.92	21.25
1:4 (25%)	0.50±0.77	10.63
1:5 (20%)	0.37±0.56	8.50
1:10 (10%)	0.13±0.64	4.25

(Data presented as mean ±SD; n=5)